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Cell Locomotion and Tumor Penetration

Report on a Workshop of the EORTC Cell Surface Project Group

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THE PURPOSE of the workshop was to evaluate the role of active locomotory movement in tumor penetration, and to consider aspects of cell locomotion and tumor penetration from morphological, biophysical, and biochemical viewpoints. Within this framework, presently available knowledge on the locomotive apparatus of the non-muscular mammalian cell was discussed.

I. EVIDENCE FOR CELL LOCOMOTION IN TUMOR PENETRATION

In histologic sections, isolated cancer cells or groups of cancer cells are often found, and in many instances the lack of continuity between such elements and a neighboring tumor was proven by means of complete sets of serial sections or even by three-dimensional reconstruction [1]. Taken together, these efforts have established the fact that displaced cancer elements surrounded on all sides by host structures do occur. Can this be accepted as proof that locomotion has taken place?

If one considers in a general way how a tumor cell may be translocated from one position in a normal tissue to another position, at least three possible mechanisms can operate:

(1) The tumor cell may "walk".

(2) The tumor cell may be relatively passive, merely forming adhesions with a normal cell whose membrane flow could transport the tumor cell until it formed new adhesions with an adjacent normal cell, breaking the adhesions with the first one.

(3) The tumor cell may be entirely passive and be pushed by a pressure gradient derived from increasing tumor cell mass or by interstitial fluid flow facilitated by muscle contraction and pulsation of blood vessels.

For discrimination among these possibilities, histology will always offer indirect evidence only. It should be admitted, however, that static morphology has its "*in flagranti*-situations" where indirect evidence of tumor cell locomotion is practically conclusive. It is the same kind of observation as is widely accepted for locomotion of leukocytes, e.g., with regard to migration of lymphocytes across the wall of venules [2, 3].

This, however, does not alter the fact that direct evidence for the role of tumor cell locomotion in penetration should be provided by

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cinemicrographic observation and recording under appropriate experimental conditions. There are limited possibilities of *in vitro*-studies which appear most promising where natural membranes, like omentum, mesentery and chorioallantois, are utilized. But even under this qualification and with a sophisticated technique, e.g., edge on-view [4, 5], penetration by locomotive tumor cells, so far, has not been demonstrated unequivocally. *In vivo*-studies are not less controversial. There are very few models allowing cinemicrography of tumor cells within compartments of the living organism, and in most instances, the traffic of tumor cells within blood vessels, but not locomotion and penetration, were recorded. In some studies of this type, egress of tumor cells from vessels was again based on indirect histologic evidence [6]. Wood [7], however, can be credited to have provided cinemicrographic documents on penetration of V2 carcinoma cells through the walls of capillaries and venules in the rabbit ear chamber. This has remained an isolated finding. It should be noted that both histologic and cinemicrographic evidence on *in vivo*-locomotion of leukocytes and tumor cells hinges upon escape from blood vessels. This may be due to special environmental conditions (see section IV). Besides, there is ample electron microscopic evidence that in many instances, egress of tumor cells after lodgment of tumor cell emboli [e.g., of the Walker 256 carcinosarcoma: 8, 9] is not accomplished by locomotion, but by intravascular proliferation, resulting in partial or complete destruction of the vascular wall and growth of the tumor focus to the extra-vascular compartment.

So far, emphasis has been laid on penetration by single tumor cells. Its most characteristic expression is infiltration of organs by leukemia cells. This process can hardly be conceived without locomotion, although direct proof under *in vivo*-conditions is lacking. Locomotion is also assumed to be involved in invasion by contiguous sheets or cords of tumor tissue. On indirect histologic evidence, this appears to be a more frequent occurrence than infiltration by single tumor cells (apart from leukemia). It proceeds along pre-existing anatomic planes or into local regions of tissue weakness created by tumor-associated pathologic mechanisms. Progress of tumor-tongues or whole tumor-fronts could conceivably be mediated by movements of cells at the advancing edge, in a manner analogous to epibolic morphogenetic movements of surface cells seen in the amphibian egg [10]. Another possibility could consist in locomotion of tumor cell aggregates. Growth in the form of isolated foci (aggregate replication) was described for a number of animal and human tumors [11]. Such aggregates are a conspicuous feature of the growth and penetration front of the V2 carcinoma of the rabbit. For this tumor it had been demonstrated that small clusters of from three to five cells moved at times as a unit [12]. In recent cinemicrographic studies [13], isolated V2 aggregates were shown to either migrate as units or to dissociate into single cells which display individual locomotion. In accordance with histologic findings, a similar behavior of tumor aggregates in the organism can be expected. Evidence was obtained that a guinea pig hepatoma penetrated into the chorioallantoic membrane as small aggregates rather than as single cells [14].

In conclusion, it can be said that there is vast indirect (histologic) evidence, but hardly sufficient direct (cinemicrographic) proof for the occurrence of cell locomotion in tumor penetration *in vivo*. There is also no direct proof for the role of pressure, fluid currents and other factors acting upon the tumor cells. It appears reasonable to assume that locomotion is of prime importance, but that passive mechanisms can be involved to different degrees in penetration of different tumors, and that different tumors use different mechanisms at different stages of their development.

II. THE CONCEPT OF TUMOR CELL MOBILIZATION

With the exception of leukocytes, the cells of the adult organism, under physiological conditions, are immobilized, though not immovable. If normal or neoplastic tissue cells begin to migrate, they must have been released from immobilization or, in other words, they must have been mobilized.

The term mobilization was originally introduced to designate changes in cultured tissues which increase the amount of migration of cells from them [15–16]. It is suggestive to transfer the concept of mobilization from the tissue culture level to the situation of the tumor *in vivo* [17].

Of the factors responsible for tumor cell mobilization, release from mechanical confinement will be considered first. Mutual attachment of the cells is achieved by specialized structures at the cell surface (junctions) and in the extracellular matrix (fibers), and by submicroscopic physicochemical bonds acting in the intercellular space. Facilitation of cell detachment, a characteristic property of some

but by no means all malignant tumors, may be due to lack of differentiation coinciding with neoplastic transformation and progression; it may also be due to degenerative processes.

Junctions in tumors can be markedly reduced in comparison to the homologous normal tissue [18], but this is by no means a general finding [19]. In mechanical terms, reduction of junctions might reduce adhesion if no other processes were involved. The loss of cell-to-cell passageways for regulators or nutrients can possibly be linked to alterations of growth control [20], or degeneration [21], and could also be conceived as a factor in cellular mobilization.

According to common experience with cell separation procedures, the contribution of enzyme-dissociable bonds to tissue stability cannot be neglected. Sylven's work [22, 23] indicates possible interrelationships of factors acting on physiochemical bonds in the intercellular compartment of tumors. Besides a host of hydrolases, lactate as a naturally occurring chelator of divalent cations may play an important role in tumor cell detachment.

Support for the suggestion that lysosomal enzymes facilitate cell detachment by means of "sublethal autolysis" [24] comes from the observations that exposure of attached cells to demonstrable lysosomal activators, including antisera and vitamin A, promotes their detachment. Studies of hypervitaminotic A animals bearing mammary adenocarcinomas revealed an association between the increased numbers of cells shaken free from excised tumors, and a higher incidence of pulmonary metastasis [25].

Sublethal autolysis is comparable to necrobiosis, which was suggested as another mechanism of tumor cell detachment [26]. The disjunction theory of local spread of cancer is based on two assumptions:

(1) Single cancer cells are detached from a tumor mass due to necrobiotic dissociation.

(2) Such elements are actively or passively translocated into the surrounding host zone where they give rise to so-called micrometastases.

It should be noted that detachment or separation of tumor cells from a tumor mass, as discussed here with respect to tumor cell mobilization, does not necessarily occur at the same plane as adhesion. However, the sequence of the two different phenomena of adhesion and separation is an absolute prerequisite for active movement of an already mobilized cell within its environment, and will therefore be discussed in section IV.

Detachment is not mobilization: It appears unlikely that detached tumor cells are automatically mobilized. The apparent state of non-attachment in which tumor cells exist in many tumors (or parts of them, e.g., at the penetration front) is not necessarily a state of non-contact. What is the general significance of contact? Although it is difficult to transfer the essentially two-dimensional *in vitro*-model of contact inhibition of locomotion to the three-dimensional *in vivo*-situation, one should tentatively accept an extrapolation to the organism, at least in the form that mobilization of cancer cells *in vivo* consists not only in detachment, but also in release from a contact-mediated restraint comparable to contact inhibition of locomotion *in vitro*.

III. PROLIFERATION AND LOCOMOTION OF TUMOR CELLS

Under physiological conditions, division and locomotion, two elementary cellular functions, are under strict homeostatic control. In the neoplastic state, these regulations are impaired, and the result is "uncontrolled" growth and locomotion, a constellation that makes up malignancy. To what extent are growth and locomotion in tumors interconnected?

Resuming the discussion on mobilization, we have to consider the role of mitosis. Decrease of attachment is a regular event during cell division. Judged from *in vitro*-experience, mitotic detachment is more conspicuous in epithelial than in mesenchymal cells, but even in epithelial cells it must not be of a complete round-about nature. The condition is that cellular cleavage is mechanically possible. For the regenerating liver, which produces mitotic cells far in excess of the most malignant tumors, it was shown that parenchymal cells can be detached more easily during a distinct part of the regeneration period [27]. It has to be assumed that cells of the regenerating liver emerging from telophase are reattached rather quickly. There may be a short delay which allows them to display the necessary positional shiftings. It is unlikely, however, that during this interval they migrate in a histologically appreciable degree. This may be due to some kind of restraint comparable to contact inhibition of locomotion *in vitro*. Such a restraint is not required while attachment is operative, nor is it necessary during mitosis, as dividing cells are unable to migrate. But it could be an immobilizing factor during the post-mitotic phase, between the end of telophase and the completion of reattachment. The situation may be similar in tumors with a relatively high

degree of cohesion. In less cohesive ones, proliferation will more or less be restricted to already detached cells, and attachment of postmitotic cells will not occur any longer, in other words: attachment as a measure of immobilization has disappeared. This reflects a permanently altered state of the cell surface inherent to malignant transformation, but does not imply that proliferation *per se* is a mobilizing mechanism.

Although more concrete data are available from investigations *in vitro*, they do not, at the moment, allow a precise disentanglement of proliferation and locomotion. Normal fibroblasts in confluent monolayers are subject to two types of control, density-dependent inhibition of growth [28], and contact inhibition of locomotion. The interdependence of the two inhibitions is a matter of debate [29]. Experimental procedures have been described that stimulate fibroblast locomotion in the absence of proliferation [30, 31], and fibroblast proliferation in the absence of locomotion [32]. Thus, under the special conditions of fibroblast monolayers at confluency, control of proliferation and of locomotion appear to be independent phenomena. No direct comparison to transformed cells with their impaired inhibitions is possible. Factors have been isolated from cultures of transformed cells that stimulate both proliferation and locomotion of normal cells [33, 34]; this means that at the molecular level proliferation and locomotion can not be separated so far. Further information will eventually be provided by Burger's present studies on effects of succinylated Con A, added to cells during mitosis and early G 1, on their subsequent locomotive behavior.

Finally, we do not know to what degree migration factors influence penetration and metastasizing. Isolated observations tell us nothing about causal relationships, and need to be repeated for many different types of metastasizing and non-metastasizing cancer in order to establish the basic correlation.

IV. THE LOCOMOTIVE TUMOR CELL IN ITS ENVIRONMENT

In studying locomotion, we are concerned with cell displacement, that is the distance between the position of a cell at the beginning and at the end of a standard period of time (such displacement is commonly not linear against time). Environmental influences on displacement can induce:

(1) non-directional responses ("kineses")

(2) directional responses ("taxes").

It is practically impossible to analyze these and most other environmental aspects of cell locomotion under *in vivo*-conditions. The bulk of the following discussion, therefore, relates to *in vitro*-studies.

A. In vitro-*studies*

A large amount of experimental work was concentrated on the processes of cellular adhesion and separation [24]. Both are of crucial importance, as any cell migrating in any environment must:

(1) Make contact with and adhere to the substratum, to obtain a "hold" against which it can "push", or "pull", or both. Adhesion cannot be measured directly and rates of adhesion are the usually determined experimental parameter.

(2) Break contact in regions of adhesion with its substratum. This process of focal separation is spatially distinct from the process of adhesion, and must be measured by different techniques.

(3) Be capable of generating the necessary locomotor energy to propel itself.

Adhesion. The substratum to which a moving cell adheres is usually provided by the solid part of the extracellular environment. *In vitro*, nonadhesive substrata can be examined; they completely stop moving cells which collide with them from proceeding any further in that direction. A continuous gradient of adhesiveness may produce a directional response; this is haptotaxis [35]. There may also be non-directional responses to adhesiveness. In general a substratum providing poor adhesion (or structurally weak) or a substratum providing excessive adhesion is likely to diminish speed of locomotion, by interfering with the normal cycle of relations to the substratum.

In order to form adhesive bonds, cell surfaces or molecules attached to them must approach within approximately 0·5 nm of each other. Although in the case of "specific" adhesions the nature of the bonds formed may well be of major importance, in the case of adhesion in relation to cell movement such specificity seems an unlikely requirement. If contact over an adequate area can be achieved, then any level of adhesive strength can be accomplished by bonds of different types ranging from comparatively weak hydrogen bonds to the high energy covalent bonds. Therefore, in connexion with the formation of the non-specific adhesions required for active cell movement, the major

problem is not how cells form adhesions, but rather how they approach closely enough to permit such adhesive bonds to be formed.

All mammalian cells and biological surfaces so far examined carry a net negative surface charge. Therefore, contact between cells and between cells and other surfaces carrying net negative charges including glass, and glass exposed to serum-containing media for example, tends to be prevented by the mutual electrostatic repulsion between them. In colloid systems, which in some important respects are analogous, whether or not contact between charged particles and subsequent flocculation or adhesion occurs, depends in general terms on the balance of the electrostatic forces of repulsion and the Van der Waals' forces of attraction, in relation to Brownian or other movements.

In computing average electrostatic interactions between cellular and other surfaces the key experimental measurement is that of electrophoretic mobility. From this, cell zeta potential and the net surface charge density may be calculated. It is emphasized that these two average quantities relate to the cellular electrokinetic surface. Attempts to predict the adhesive, or contactmaking behavior of cells on the basis of average estimates of the potential energy barriers between them have been largely unsuccessful [36]. From this failure, it may be reasonably concluded that the physical bases of cell contact depend on non-average or heterogeneous properties of the cell periphery. Electron microscopic data on the adsorption of positively charged markers to a variety of cells support the view that negative charges are indeed distributed in clusters at the cell periphery, and that cell projections find and/or create gaps in these clusters in order to make contact with another cell in regions of lower-than-average charge density.

It is probable that potential energy and viscous barriers tend to prevent cells from making contact with, and adhering to, other cell surfaces. Without such contact and adhesion, active translatory movements of cells cannot occur. The suggestion that cells make contact in spite of these apparently insurmountable average barriers by making contact through comparatively small, non-average surface regions is compatible with observations on the comparatively small fractions of the total surface areas of the opposed surfaces of many different types of cells involved in making tight junctions, and in junctions of low electrical resistance.

Separation. Unless the regions by which a cell adheres to a substratum are infinitely extensible, it follows that some sort of separation process must also be associated with adhesion-dependent movement. Whereas detachment of cells from solid tumors involves breaking all their contact regions with the primary lesion, active cell movement does not require the simultaneous breaking of all contact regions associated with the cell periphery in question, but may well be a very localized sub-cellular phenomenon. Although environmental enzymes could contribute to cell separation in relation to active movement, they could also destroy or prevent cell/substratum adhesions by virtue of bond fission at the adhesion interface. While the former activity could promote active cell movement, the latter would effectively prevent them.

Apart from cellular adhesion and separation, *in vitro*-studies on environmental conditions of cellular locomotion have drawn our attention to two further constellations, contact guidance, and contact inhibition of locomotion.

Contact guidance. This is another way by which the extracellular substratum can affect locomotion. It is a directional response for which the form of the substratum, not variations in its adhesiveness, is effective. Contact guidance, on principle, applies both to normal and malignant cells, but at least in the latter, it is by no means of regular occurrence. In cinemicrographic studies on the locomotion of rat leukemia cells and V2 carcinoma islands on mesentery maintained under tissue culture conditions, locomotion of both the single tumor cells and the tumor cell groups took place, after deterioration of the mesothelium, on the submesothelial network of collagen and elastic fibers, but without any expression of contact guidance [13].

Contact inhibition of locomotion. How do locomotive cells react to collisions with other cells? Migrating fibroblasts in culture, the most studied from this point of view, have a special reaction, not so far shown in any encounter with a non-cellular object. This is contact inhibition [37], which, it must be emphasized, is a directional response. A cell whose front-end has made contact with another cell is prevented from continuing its locomotion in that direction. The cell can however move in a different direction. The reaction is easily distinguishable from the block to movement that occurs when a cell collides with a non-adhesive patch of substratum.

The reaction is not confined to fibroblasts. Epithelial cells show something very like fibroblast contact inhibition. There is a reaction

between malignant cells of many kinds which at least has the same outcome as contact inhibition between fibroblasts though precise details are as yet unstudied. It is often said that contact inhibition between malignant cells is deficient. The effectiveness of the homologous contact inhibition (i.e. between like cells) was estimated in a range of human malignant cells by an indirect method (overlap index), and much greater variation was found than in normal fibroblasts, many malignant cells having a lower intensity of contact inhibition, but some a higher [38].

Since *in vivo*, malignant fibroblasts invade populations of normal fibroblasts, heterologous contact inhibition (between unlike cells) is of special interest. It has usually been found to be defective in comparison with the homologous inhibition of normal fibroblasts [37–39], but not always [42]. Malignant fibroblasts, advancing as whole cultures and as detached single (sentinel) cells, were completely stopped when opposed by coherent and compact sheets of normal epithelial cells [43, 44].

B. In vivo-*studies*

It is evident that all extrapolations from *in vitro*-studies to *in vivo*-conditions are fraught with the danger of misinterpretation, one major obstacle being the difference in intercellular connection and organization between monolayers *in vitro* and tissues *in vivo*. To begin with: It is very difficult to describe morphologically the compartment in which cells migrate *in vivo*, to analyze chemically the influence of fluid and solid constituents of the extracellular matrix on locomotive cells. Judged from electron micrographs, leukocytes must possess a very smooth and efficient mechanism of locomoting through the ground substance; they are, for instance, able to pass the basal lamina without producing the slightest alteration [45]. As regards penetrating tumor cells, there is morphological evidence, at the light and electron microscopic level, for both possibilities: lack of lytic action [46] as well as slight to massive destruction of host tissues in the immediate invasion zone. Sylven mentioned the important role of edema at the tumor periphery, which leads to opening up of tissue spaces, thus facilitating the access of tumor cells into preformed tissue spaces. The degree of edema is characteristically correlated to the grade of malignancy and largely influenced by the rate of tumor glycolysis [47]. Pathologists are liable to forget this fluid phase, since it is not seen in their sections and electron micrographs.

It thus appears that on principle, tumors are able to create either directly or indirectly a "penetration space" which enables or facilitates the onset of cellular locomotion. Lysosomal hydrolases may, therefore, not only play a role in cell detachment, but also in penetration [48]. Sylven's earlier work [49, 50] was directed to the fluid interphase of tumors, and separate sampling was undertaken both of interstitial fluid from the tumor periphery as well as the necrotic central regions. Very marked increases in activity of lysosomal hydrolases were noticed but also many other enzymes, present in the soluble part of the cytoplasm, were found. It is clear that added enzymatic components in the tumor compartment can only derive from local sources, either from the tumor cells or host cells. Since the number of host cells is relatively small in solid tumors, we are left with the suggestion that the enzymatic components are derived from tumor cells. This suggestion is strongly substantiated by incubation studies of ascites tumor cells [51]. Living ascites tumor cells were incubated in a full growth medium for short times and the release of enzymes to the medium was studied. The tumor cells were found to leak out or secrete very considerable amounts of both lysosomal and other cytoplasmic enzymes.

The greatest interest regarding these enzymes is focused on proteinases. Neutral proteinases and their activators have been demonstrated in human tumors [52–54], together with the lysosmal enzymes cathepsin D and B. Both lysosmal enzymes have acid pH optima but the most likely candidate in the present context seems to be cathepsin B, since its activity extends up to pH 7.5 [55].

Immunohistochemical studies on the localization of lysosomal cathepsins have been hampered by many methodological difficulties. Recent work on the localization of cathepsin D by antibody markers indicates that at least in some tumors, extracellular enzyme is detectable at their edges and in the surrounding stroma [56]. Thus, the origin of this enzyme could be from the tumor and/or the stroma. In the case of breast neoplasms, both (benign) fibroadenomas and carcinomas may secrete large or small amounts of both proteases, and there is therefore no correlation between enzyme secretion and malignancy or invasion.

It is possible that tumors exert their degradative effects through interactions with nonmalignant cells including fibroblasts, endothelial, lymphoid and monocytoid cells and platelets. In addition, other agents must also be considered. Hypercalcemia in rats having bone destruction due to injected Walker tumor cells was abolished by administration of the anti-

inflammatory, non-steroidal drugs, aspirin and indomethacin, which are used in the treatment of osteoarthritis [57]. As these drugs are known to act mainly by their inhibition or prostaglandin synthetase activity, it is possible that tumor-induced osteolysis is mediated by prostaglandin release from tumors.

In a partly liquefied tumor-host interzone, the fibrinogen–fibrin system may play a particular role. Fibrinogen, as a constituent of the interstitial fluid, can be converted to fibrin by thromboplastin-like agents deriving from the tumor as well as from the host. Fibrin has been claimed to provide contact guidance to tumor cell locomotion, but it has also been credited with a barrier function similar to the one displayed in the spread of certain infections. At any rate, the fibrinogen–fibrin system with its great versatility, based on thromboplastic and fibrinolytic properties of both tumor and host, should be considered a key factor in the events at the penetration front [58].

In recent reports, it has been demonstrated that the upper surface of cultured fibroblasts and epithelial cells lacks adhesivity for other cells [59–60]. These findings have been confirmed for a variety of cells [61]. Macrophages from the rat abdominal cavity, for instance, were found to be unable to spread on rat mesentery. It may be assumed that the particular configuration of cellular surfaces, e.g., the presence of microvilli, prevents other cells from adhering and spreading. On the other hand, the inner surfaces of blood vessels and body cavities are frequent sites of tumor cell adhesion and, therefore, important starting points of penetration. Two mechanisms, acting singly or combined, can accomplish the adhesion of tumor cells to these surfaces:

(1) Microcoagulation around tumor elements.
(2) Exposure of subjacent connective tissue strata after retraction of endothelial and mesothelial cells.

Among the vast literature on the role of microcoagulation in tumor cell lodgment, studies on adhesion of tumor cells to vein walls are particularly relevant [62]. Intravenously injected lymphoma and carcinoma cells first became surrounded by platelets and then adhered to the wall of the inferior vena cava in two ways which were characterized by the presence or absence of damage to the underlying endothelium. On intact endothelium, there was loose adhesion of tumor cell-platelet-emboli with negligible production of fibrin, and these emboli deteriorated rapidly. On the other hand, on areas with damaged or absent endothelium, the tumor cell-platelet-emboli became firmly embedded in fibrin, and progressive passage of the tumor cells through the fibrin layer and the vessel wall could be demonstrated. It is, therefore, possible that in this experimental model the fibrinolytic activity of intact endothelium impedes adhesion and subsequent locomotion of tumor cells.

Retraction of endothelial or mesothelial cells, an interesting phenomenon of cellular motility in itself, can lead to the exposure of the deeper layers, basal lamina and connective tissue. Tumor cells exclusively or preferentially adhere to these denuded zones, as has been shown by scanning electron-microscopy for various tumors implanted into the peritoneal cavity [63–66]. While the exact causes of mesothelial retraction are unknown, some indications for endothelial cells were given concerning the contraction-inducing effect of histamine-type mediators [67] and the corresponding effect of thrombin [68]. Thus, tumor cells obviously are able to mobilize various agents that help them to bypass the relatively nonadhesive endothelial and mesothelial surfaces.

Without doubt, many other factors must be assumed to be operating on locomotive tumor cells in the ecosystem constituted by the tumor-host interzone. Nothing is known, for instance, about effects of antibodies, directed against surface antigens of tumor cells, on the latter's locomotion.

V. THE LOCOMOTIVE APPARATUS OF THE NON-MUSCULAR MAMMALIAN CELL

From a chemical viewpoint, close similarities exist between the proteins involved in contraction of striated muscle and contractile proteins present in non-muscular cells. It is therefore a reasonable assumption that this group of proteins, the actomyosin system, is responsible for movement in both muscular and non-muscular cells. Thus, locomotion of tumor cells can also be expected to depend on the operation of the actomyosin system.

In muscle cells, actin and myosin appear as filaments arranged in segmented arrays, and motive force generation is due to a sliding mechanism of these filaments past each other, driven by repetitive interactions of cross-bridges between them. An exploration of the locomotive apparatus of non-muscular cells, therefore, had to start with an inventory of filamentous structures.

Electron microscopy, so far, indicates the

presence of three types of filamentous organelle in eukaryotic cells:

(1) microfilaments with an approximate diameter of 6 nm

(2) a collection of filaments with diameters of 8–11 nm, called "100 Å filaments"

(3) microtubules: hollow cylinders with a total diameter of 25 nm and an empty channel of 15 nm and composed, most likely, of 13 individual protofilaments.

What is the relation of the actomyosin system to these filamentous structures?

Microtubules consist mainly of tubulin, a structural protein which contains two different polypeptide chains with a molecular weight of 53,000 daltons each. It appears, therefore, that microtubules are not directly linked to motive force generation based on the actomyosin system.

Of the two classes of filaments, the 100 Å filaments, so far, have resisted all attempts of chemical analysis: they are, in biochemical terms, an unknown system.

The situation is different with microfilaments. Heavy meromyosin (HMM) is a specific histochemical stain in electron microscopy for microfilaments, giving rise to characteristic double arrow-headed structures [69]. HMM is the proteolytic headpiece of the myosin molecule which can form specific complexes with polymerized actin, the F-actin. Thus the easiest explanation for the decoration experiment is that F-actin like polymers are the major structure of the microfilament.

More information on actin as a constituent of microfilaments was obtained by the recent introduction of an immunofluorescence microscopy assay [70]. It allowed the demonstration, within cultivated mouse 3T3 cells and a variety of fibroblasts and epithelial cells from different species, of a complex array of straight actin-containing fibers with the following properties:

(1) The actin fibers show no interruptions. The diameters vary within the same cell and especially between different cell lines. In some fibroblasts diameters of 1 μm can be reached.

(2) Different cell lines show a high degree of individuality in pattern formation.

(3) The fibers run often parallel to each other along the long axis of the cell.

(4) The fibers converge at some focal points. The expression of focal points can differ with the cell line.

(5) The fibers are concentrated at the inner side of the plasma membrane as submembraneous bundles.

Furthermore, locally concentrated but not substructured actin organization could be detected within ruffling membranes.

The next step consisted in the demonstration of myosin localization and, here again, the approach by immunofluorescence microscopy was successful. Myosins from non-muscle cells cross-react with smooth-muscle myosin, and it could be shown that antibody against gizzard myosin decorates the same fibers in fibroblasts that are decorated by the actin antibody [71]. There is, however, one important difference: myosin antibody gives rise to interruptions and striations whereas actin antibody gives uninterrupted fibers. Thus, as expected from the chemical features of myosin, the molecule is interspersed into the actin fibers most likely in forms of small oligomers.

This result clearly invites the interpretations that:

(1) myosin is localized close enough to F-actin within the same microfilament system, and that

(2) this localization allows the minimal requirement for the classic principle of actin-myosin interaction as a basis for potential contractility.

Following this first success in the biochemical anatomy of microfilaments, it was shown that other accessory proteins like tropomyosin and α-actinin are also part of the microfilament [72]. So it seems that microfilaments are not a storage form of F-actin but contain the whole contractile apparatus.

On this basis, it appears legitimate to use the term actomyosin filament or fibril for those structures in non-muscular mammalian cells that can be assumed to be directly involved in contractility and motility. That this is indeed the case was demonstrated for stress fibers (=thick bundles of microfilaments) of a fibroblastic cell line derived from a rat mammary adenocarcinoma [73]. Single fibers were cut out from lamellipodia of the spread cells by a laser microdissection technique, and active shortening of the isolated fibers, in presence of ATP and calcium, could be recorded by cinemicrography and studied by single frame analysis.

Thus, the actomyosin fibril of non-muscular mammalian cells has been recognized as a morphological as well as a functional unit of contractility. The role of this unit with regard

to cellular locomotion, however, is far from being clear. In contrast to the contractile proteins in muscle sarcomeres, the cytoplasmic actomyosins have not only the task of transforming chemical energy into mechanical work, they have also to provide the possibility of being themselves translocated over relatively long distances during most cytoplasmic movements, particularly so during locomotion. The transformation of F-actin to shorter chains or to G-actin and vice versa is an obvious advantage, if not a precondition, for such translocations of the contractile system: a transport of G-actin or short-chained actin is presumedly easier to perform than the translocation of F-actin which is a linear molecular aggregate with an extreme length in the μm-range.

In connection with these translocations and with locomotion as a whole, changes of the cytoplasmic consistency are indispensable. Work with slime molds and amoebae [74–76] offers a model of cytoplasmic events that, in all probability, is applicable to every type of locomotive cell. In Physarum as well as in many free living amoebae, there is a permanent transformation between the low viscous endoplasm and the higher viscous, i.e. rigid ectoplasm (sol$\rightleftharpoons$gel transformation). Actin in the flowing endoplasm is largely in the depolymerized state and transforms to F-actin spontaneously within the aging endoplasm. Therefore, it seems to be evident that G$\rightleftharpoons$F-actin transformations are involved in the cyclic change of endoplasm$\rightleftharpoons$ectoplasm in Physarum. There is a dynamic equilibrium between filaments (F-actin) and subunits (short-chained actin or G-actin). Cytologists in the first half of this century paid much attention to cytoplasmic viscosity changes and the "contractile gel-reticulum" of the cell. Today, there is hardly any doubt that the "contractile gel-reticulum" can be identified as cytoplasmic actomyosin, and that viscosity changes can be explained by the known ultrastructural and biochemical properties of cytoplasmic actomyosin.

On this basis, it is not unreasonable to assume that also in non-muscular mammalian cells, the actomyosin system is involved in both volume contraction of the cell body (under isotonic conditions) and in internal stabilization (under isometric conditions), and that these interconnected functions are linked to the non-fibrillar and fibrillar state, respectively, of the contractile proteins.

The concentration on microfilaments should, by no means, express the opinion that 100 Å filaments and microtubuli are not integral parts of the locomotive system. Recent electron microscopic studies in leukemias [77] have shown strikingly different distribution patterns of 100 Å filaments in resting and locomotive cells. The functional significance of this observation, however, is unknown, and the same statement must be made with regard to microtubili, although both organelles are often assumed to act as a cytoskeleton.

There is a very considerable body of knowledge on the nature of the contractile proteins in a wide variety of cells. However, the relationship of these data to cell movement and cell infiltration is unknown. The problem can perhaps be clarified by means of a simple analogy. If we consider a motile cell to be analogous to a car, the question is almost: How much can one predict about the car's speed, performance, direction and "social" behavior with respect to other cars, from inspection of its engine? The answer is: very little! Although we could deduce from comparison of their engines that a racing-car is faster and has more potential for acceleration than a 2-CV, providing their transmission systems are working, how fast they go in traffic between two points depends on the nature of the road—is it greasy, wet or dry?—stop signs, pedestrian crossings etc. and, above all, the driver. In a similar manner, the locomotion of cells cannot be predicted solely from examination of their contractile elements, but the substrata they move over or through must also be taken into account, as indeed must internal and external controlling mechanisms.

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Variations of Liver Cell Control During Diethylnitrosamine Carcinogenesis

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Abstract—*Male Wistar rats chronically treated with diethylnitrosamine (DENA) (80 mg/l in drinking water) develop liver carcinomas mostly after the 90th day of treatment.*

Experiments were planned in order to investigate the cell kinetics of liver during the preneoplastic period (up to the 60th day). The mitotic response of the liver was studied in various conditions: in animals treated with DENA alone and in DENA treated rats submitted to a partial hepatectomy. Furthermore, normal hepatectomized rats were treated with liver extracts obtained in various conditions (normal and DENA treated rats). The extracts were prepared in order to purify mitotic inhibiting substances (chalone activity) normally present in liver tissue.

During the two first weeks of treatment, DENA induces a transient increase of mitotic index; after partial hepatectomy, the mitotic response is approximately normal. The cell divisions present during this period a normal circadian rhythm.

After the 30th day of treatment, the mitotic index decreases significantly, the response to the partial hepatectomy is reduced and the circadian rhythm of divisions is lost. During this period, glycogen retaining areas develop in the liver; no difference is observed between the response of these areas and that of adjacent parenchyma.

An extract obtained from a normal liver inhibits to a large extent the ^3H-thymidine DNA label and the mitotic response induced by a partial hepatectomy. A similar extract obtained from the liver tissue taken 24 hr after hepatectomy has no inhibitory effect. Extracts from liver of rats treated during 30 and 60 days with DENA behave in the same way as an extract from regenerating liver and produce no inhibition.

The results indicate that the preneoplastic period shows at least two successive phases. During the two first weeks, the mechanisms controlling the cell homeostasis of liver tissue seem to function normally. Later on, there seems to be an important disturbance of these mechanisms.

INTRODUCTION

RATS continuously fed with a diet containing diethyl- or dimethylnitrosamine develop a high percentage of liver carcinomas [1]. The appearance of the tumors is preceded by a latent period the length of which varies according to the experimental conditions. It has been shown that during this preneoplastic period, enzymic changes occur in circumscribed areas of the liver parenchyma. The decrease of glucose-6-phosphatase and adenosine triphosphatase activity in these areas is well documented [2, 3].

Very little is known about the disturbances of the organ specific mechanisms which control the cell proliferation and function. It is likely that these homeostatic mechanisms undergo also changes during the preneoplastic period. It is possible to investigate the homeostatic regulation by measuring the proliferation rate of liver cells after partial hepatectomy. Rabes *et al.* [4] have followed the changes of labelling index and enzymic content of liver cells during the latent period in diethylnitrosamine (DENA) treated rats. They have described three successive phases in the liver response:

(1) Period of weakening of the activities of glucose-6-phosphatase and adenosine triphosphatase activity of acinocentral hepatocytes coincidently with loss of ability to proliferate even after organ specific stimulation of growth;

(2) Period of complete loss of the activities of glucose-6-phosphatase and adenosine tri-

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phosphatase within circumscribed acinocentrally localized groups of hepatocytes, coincidently with return to ability to proliferate, but for almost all hepatocytes of the enzyme deficient cell groups only after specific stimulation by partial hepatectomy;

(3) Period of autonomous proliferation of these enzyme deficient cells without an additional growth stimulus, accompanied by a loss of response to organ specific homeostatic control."

There is good evidence also that the tissue homeostasis is controlled by substances released by differentiated cells. These so-called "chalones" [5] depress specifically the mitotic activity in the tissue from which they are extracted. It is possible to prepare a liver extract which depresses specifically the mitotic activity in hepatocytes after partial hepatectomy [6–9].

The aim of the present work was to study the influence of DENA treatment on the mitotic response following partial hepatectomy and on the "chalone activity" of liver tissue. The attention was focused on the preneoplastic period extending up to the 60th day of treatment. During this period, nodular areas appear in which glycogen is retained after fasting owing to enzymatic disturbances.

On the other hand, previous results have shown that the mitotic activity induced by partial hepatectomy varies significantly in the course of the day [10–14]. This circadian rhythm of mitoses which is also part of the tissue homeostasis has to be taken in account in planning the experiments.

MATERIAL AND METHODS

Male Wistar rats, weighing approximately 180 g, were treated chronically by diethylnitrosamine (DENA) given in drinking water (80 mg/l) which represents an ingested dose of about 10 mg/kg. The experiment was divided into two parts. The first was devised in order to test the ability of hepatocytes to divide at different stages of the preneoplastic period: 7th, 15th, 30th and 60th day after beginning DENA administration.

At each delay, 5 treated animals were sacrificed at 10 a.m. and 5 at 10 p.m. At the same time, 10 animals were submitted to a partial hepatectomy [15] at 10 a.m.; among them, 5 were sacrificed at the 48th (morning) and 5 at the 60th (night) post-operative hour. The mitotic indices were calculated for all experimental groups and were compared to those of control hepatectomized animals, not receiving DENA.

In order to rule out an acute toxic effect of the drug, a supplementary group was treated by DENA during 50 days and then left 10 days without treatment before being hepatectomized in the same conditions.

After 30 and 60 days of treatment, the mitotic activity was also measured in the "hyperplastic nodules" visible by glycogen storage after 18 hr fasting. Animals have been used as control to test the eventual influence of fasting on the liver cell response to hepatectomy. The hyperplastic areas were stained by P.A.S. after freezing-substitution fixation.

The second part of experiments was planned in order to measure the "chalone" activity of liver tissue during the preneoplastic stage.

At the 15th, 30th and 60th day of DENA treatment, 20 rats were sacrificed. In each group, hepatic extracts were prepared according to the method described by Verly *et al.* [8]. The 105·000 G supernatant of liver homogenate is treated by ethanol and the precipitate obtained between a 70 and 87% of ethanol concentration is used. This extract was diluted in saline and injected (1 ml i.p.) in normal hepatectomized animals at the 1st and 6th post-operative hour; the amount of product injected corresponded to the amount extracted from 70% of the whole normal hepatic mass. In order to estimate the "chalone" activity, the ^{3}H-thymidine labelling index and the mitotic index were measured respectively at the 24th and 28th postoperative hour and compared to the same indices found in control hepatectomized rats treated with saline solution instead of liver extract.

The results were also compared to those obtained when the extract is prepared from a normal and a regenerating liver. In the last case, the animals were sacrificed at the 24th postoperative hour at the moment of the DNA synthesis peak [11, 12]. The labelling index is measured 1 hr after administration of ^{3}H-thymidine (2 μCi/g i.p.).

All labelling and mitotic indices given in the tables are the mean of counts made in at least 5 animals. The standard error of the mean has been calculated.

RESULTS

1. *Response of liver tissue to hepatectomy*

The results of the first group of experiments are summarized in Table 1.

In the control unoperated rats, either on a

Table 1. *Mitotic activity induced by partial hepatectomy in various conditions. Results are expressed in number of mitoses/*1000 *nuclei*

	Unoperated animals			Post-operative delays			
				48 hr		60 hr	
	Normal parenchyma		Glycogen retaining nodules	Normal parenchyma	Glycogen retaining nodules	Normal parenchyma	Glycogen retaining nodules
Killing time	10 a.m.	10 p.m.	10 a.m.	10 a.m.	—	10 p.m.	—
Normal control	0	0	—	24·0(±2·0)	—	7·0(±2·0)	—
Fasting control	0	0	—	23·0(±2·0)	—	8·0(±1·3)	—
DENA for 7 days	0·5(±0·1)	0·4(±0·2)	—	26·0(±2·0)	—	6·2(±0·6)	—
DENA for 15 days	2·7(±0·5)	0·2(±0·1)	—	18·3(±2·3)	—	7·4(±1·0)	—
DENA for 30 days	0·6(±0·2)	1·2(±0·3)	1·0(±0·3)	11·0(±0·7)	6·0(±2·0)	10·3(±1·3)	6·1(±2·8)
DENA for 60 days	1·2(±0·2)	0·8(±0·2)	1·2(±0·4)	8·3(±2·0)	8·9(±2·1)	5·7(±2·3)	6·0(±2·5)
50 days of DENA treatment, interruption and killing 10 d. later on	0·2(±0·2)	0·24(±0·1)	—	5·6(±0·9)	—	5·1(±0·7)	—

normal diet or after a 18 hours fasting, mitoses were not detected.

In unoperated but DENA-treated animals, numerous necrotic foci develop and mitoses appear in the liver; the mitotic index reaches a maximum after 15 days. At this time, a circadian rhythm of mitoses is clearly visible the mitotic activity being higher at 10 a.m. When the DENA treatment is prolonged for 30 or 60 days, the mitotic activity is stabilized at rather low values and the circadian rhythm is lost. When the animals are treated during 50 days and kept at a normal diet for 10 more days, the mitotic activity is significantly lower than that found in rats receiving DENA until sacrifice at day 60.

In normal animals, a partial hepatectomy (at 10 a.m.) triggers the mitotic activity. The mitotic index presents a higher value during day time (48th post-operative hour) than during night (60th post-operative hour).

No difference is observed in regenerating liver between normally fed and fasting animals.

After 7 days of DENA feeding, the response of liver tissue to partial hepatectomy is normal. As the DENA treatment progresses, the hepatectomy triggers fewer and fewer mitoses and the circadian rhythms gradually dissappears.

Glycogen retaining nodules could be detected 30 days after beginning DENA administration. The results indicate that the mitotic activity in those areas is not statistically different from that observed in the adjacent liver parenchyma. This is true for hepatectomized rats as well as for unoperated animals.

When the drug administration has been interrupted at day 50, i.e. 10 days before the hepatectomy, the results obtained are similar to those observed after a continuous feeding of DENA for 60 days.

2. *Variations of "chalone" activity in liver during DENA treatment*

The Table 2 summarizes the changes of activity when the extracts are obtained from livers in various experimental conditions. This activity is estimated by the degree of inhibition of mitoses in normally regenerating liver.

The extract from a normal liver has a strong inhibitory effect on the mitotic and labelling indices measured in the normal liver after partial hepatectomy. On the contrary, the extract from a regenerating liver has no inhibiting effect.

When the extract is obtained from animals treated by DENA for 15 days, the inhibitory effect is still present. However, when the extract originates from liver of animals treated for 30 or 60 days, there is no more "chalone" activity to be detected. In all the experiments, mitotic and labelling indices change in parallel.

DISCUSSION

The results described in the present paper indicate that the normal control of liver homeo-

Table 2. "Chalone" activity of liver extracts in various experimental conditions. The preparations are injected to normal hepatectomized rats. Labelling and mitotic indices are measured respectively 24 and 28 hours after operation

Product injected	Labelled nuclei/1000 cells measured at the 24th post-operative hour	Mitotic index measured at the 28th post-operative hour
Saline	258(±24)	22(±2)
Normal liver extract	60(±14)	4(±1)
Liver extract after 15 days DENA feeding	24(±5)	14(±2)
Liver extract after 30 days DENA feeding	210(±20)	16(±2)
Liver extract after 60 days DENA feeding	248(±22)	22(±2)
Extract of regenerating liver (24th post-operative hour)	265(±34)	30(±4)

stasis is disturbed in rats continuously fed with a diet containing DENA. This change is revealed by the reduced ability of liver tissue to respond by a mitotic wave to a partial hepatectomy and by the absence of a circadian rhythm in this reduced mitotic activity. According to previous results of Rabes *et al.* [4, 16], such a loss of the normal control can be demonstrated long before the development of liver cancers. In fact, the liver cells seem to respond normally to a partial hepatectomy during the two first weeks after beginning DENA feeding. After a 30 day treatment, the mitotic response is already significantly decreased. It is striking that at the same time, the circadian variations of the mitotic activity have completely disappeared. Whereas in normal liver the mitotic response is higher during day time than during night time, the mitotic indices found in DENA treated animals are the same whatever the moment, day or night time. It is noteworthy that there is no significant difference between the response of the normal looking tissue and the glycogen retaining areas which are detectable after the 30th day.

The DENA feeding induces by itself a certain mitotic activity in response to the liver damage. Up to day 15, the mitotic index shows a circadian rhythm with a higher peak at day time.

After a 30 day treatment, the mitotic activity decreases, as compared to a 15 day treatment. This could indicate that the number of cells able to respond by mitoses to the DENA damage decreases. It seems however that these cells still able to respond to the stimulus inducing mitosis have escaped the circadian control. This could be a consequence of a toxic effect of DENA given in rather large dose. When DENA treatment is interrupted at day 50, the mitotic activity is still decreased 10 days after this interruption. This observation is compatible with a possible decrease of cell destruction and hence a reduction of the mitotic stimulus.

If a small number of cells seem still able to divide the largest part of the liver cells have not recovered their ability to respond normally to a partial hepatectomy. The loss of homeostatic control cannot be ascribed to an acute toxic effect of DENA on liver cells. The damage induced does not disappear after a 10 day interruption of DENA administration. It is likely that a DENA treatment of a certain duration induces a more permanent damage.

It seems that in our experimental conditions the preneoplastic period can be divided at least in two different phases. During the first one, lasting about 15 days, the liver tissue remains under its homeostatic control; in the second phase, this control is getting less and less effective.

There is good evidence that neuro-hormonal factors play an important role in modulating the circadian variations of mitosis [5, 14, 17, 18]. It could be that in DENA treated animals, either the neuro-hormonal activity is disturbed or the dividing liver cells escape its control. There is yet no evidence to support any of these possibilities.

As far as the induction of mitotic activity is concerned, an important role is ascribed to the suppression of the so-called "chalone activity". It is thought that substances produced by the differentiated cells have an inhibitory effect on the mitotic activity. Our results show that

inhibitory factors can be demonstrated in normal but not from regenerating liver. This suggests that at least in normal liver such factors are produced by the cells when in G_0 but not by the cells engaged in a cycle.

When the "chalone" activity of liver parenchyma is measured during DENA treatment, it appears that this activity decreases when the treatment progresses. A preparation obtained from a liver exposed to a 30 day DENA treatment has lost most of its inhibitory activity. It is easy to understand that the removal of a large part of such a liver does not influence anymore the response in the remaining hepatic tissue. One may wonder why the amount of inhibitory factors decreases during chronic DENA feeding. This could be due to a direct toxic effect of the carcinogen on the liver cells in G_0. Another possibility could be that in such livers, the number of cells entering a cycle is sufficient to reduce significantly the "chalone" production. Preliminary results indicate that during the DENA treatment, a progressive endo-polyploidization of the liver cell nuclei takes place [19]. This observation, as well as the presence of cells with intermediate DNA values suggests that a DNA synthesis is going on [20]. The cells undergoing such changes are engaged in an atypical cell cycle and it is likely that they are unable to produce the factors responsible for mitotic inhibition. Further experiments are needed to confirm this hypothesis.

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Evaluation of Routine Pre-Operative Bone Scintigraphy in Patients with Breast Cancer

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Abstract—*A prospective study on bone scanning was undertaken in patients with a clinical operable breast carcinoma to investigate its use in the staging of these patients. From 90 patients with a proven breast carcinoma, stages T_1, T_2, N_0, N_{1a}, the pre-operative whole body skeletal scintigraphy was positive in 7 cases. Four of these could be excluded because other reasons than metastases could be demonstrated to be the cause of the positive scan.*

So in only 3 cases (3·4%) the scan was positive, suggesting bone metastases.

These lesions could, up to now in no way be proven to be metastases. The final percentage of pre-operative detection of bone metastasis after a longer follow up period may therefore even be less than 3·4%. We therefore decided to omit routine pre-operative bone scanning in patients with a breast carcinoma stages T_1, T_2, N_0, N_{1a}.

INTRODUCTION

BONE scintigraphy has been performed for 15 years and in that time has become widely used as a diagnostic aid in many branches of medicine. During the past few years many changes and technical improvements have been introduced both in the field of radiopharmaceuticals and detection equipment.

Originally examinations were cumbersome, involving the use of Strontium^{85} or Fluorine^{18}, but these have now been replaced by modern radiopharmaceuticals such as technetium^{99m} labelled diphosphonates [1–6]. Simultaneous improvements have also occurred in detection technology, so nowadays skeletal scintigraphy examination by gammacamera [7] or dual probe rectilinear scanner is usually completed more rapidly than an equivalent conventional radiological examination of the same structures. In the presence of metastases, destruction and new bone formation occur simultaneously. New bone formation occurs by deposition of hydroxyapetite crystals in a matrix of osteoid tissue and the labelled diphosphonates, used in scintigraphy are bound to these hydroxyapetite crystals. This occurs at an early stage at the time conventional radiological examination reveals no abnormalities. In world literature there is general agreement concerning the superiority of skeletal scintigraphy over skeletal radiological examination for detection of metastatic bone lesions [8–12].

These facts encouraged us to undertake a prospective study of the value of bone scanning as a routine pre-operative investigation to help in the staging of patients with breast cancer. It has been estimated that about 30% of patients treated by radical mastectomy die within 5 years from direct effects of their carcinoma and in 71% of these, bone metastases can be found at autopsy [8]. Mutilating operations such as Halsteds mastectomy remain the operation of choice in cases of operable breast cancer. If bone metastases could be demonstrated in an early stage unnecessary mutilating surgical treatment would be avoided.

Accepted 24 August 1976.

MATERIAL

In the Antoni van Leeuwenhoek Ziekenhuis and the St. Lucas Hospital between October 1974 and January 1976 all patients with palpable breast tumors, clinically suspected to be carcinoma in stages T_1, T_2, N_0, N_{1a} were investigated. In all cases a conventional radiological examination of skull, thoracic cage, spine, pelvis and extremities was performed.

A skeletal scan was performed 3 hr after i.v. administration of 15mCi of ^{99m}Tc-diphosphonate (Osteoscan). The scans were made in 43 patients with an Elscint Dual Probe Whole body scanner equipped with 55-hole 90 mm-focal-length collimator (FMHW 11 mm) and in 47 patients with a Toshiba Jumbo Gamma-camera G.C.A. 401 with a 42·000-parallel-hole-resolution collimator (FWHM 8·1 mm on 10 cm). The results of the analog scans were recorded as negative, when no abnormal accumulations of radioactivity were seen, positive when there were clearly discernable hot-spots suggesting bone metastasis and equivocal when only slight or questionable local augmentations of radioactivity were seen. From every abnormal scan, positive or equivocal, extensive radiological examination was done and whenever possible a direct biopsy was performed.

RESULTS

Our series involved 110 patients (age 27–80 years). Twenty patients proved on histological examination to have benign breast lesions. From the remaining 90 patients an abnormal scan was obtained from 14, of which 7 were recorded as equivocal. In the 7 patients with equivocal scans there was no radiological or clinical evidence of metastasis and in some there were signs of degenerative bone lesions. The other 7 patients had hot-spots suggesting bone metastases. In one patient the positive scan was due to a simple healing ribfracture, confirmed by X-rays and open biopsy. A second patient with a positive scan of the lumbar vertebra's had radiological signs of severe degeneration of the lumbar spine, which was considered to be the cause of the positive scan. This opinion was strengthened by a second scan six months later, which showed no increase in activity. A hot-spot of the right maxillary sinus of the third patient was later shown by sinus tomography to be caused by pansinusitis. The fourth and fifth patient underwent biopsy for positive lesions in the lumbar spine and tenth rib and lumbar spine respectively. These lesions were normal on radiological examination. The biopsies were histologically negative for tumor although the pathologist considered one biopsy showed early signs of Paget's disease. Indeed review of appropriate radiographs tended to substantiate this diagnosis.

The remaining two patients with positive scans had hot-spots, one in the left mandible and the other in the fifth lumbar vertebra.

In these cases no biopsies were taken for various reasons, but X-ray tomography was negative. No biochemical changes indicating bone metastases were obtained in any of the patients.

DISCUSSION

From 90 patients with histologically proven breast carcinoma clinical stages T_1, T_2, N_0, N_{1a}, whole body skeletal scintigraphy revealed in 7 cases a hot-spot, suggesting bone metastasis. In four of these a benign lesion could be demonstrated to be the cause. The remaining 3 cases (3·4%) had a hot-spot, not otherwise explicable, suggesting a bone metastasis, but these could not definitely be proven to be so. This percentage is a maximum because with a longer follow up period some of these lesions may show not to be caused by metastases after all. Statistically our results mean that the chance a hot-spot suggesting bone metastases may be found on scanning, in a patient with breast cancer stages T_1, T_2, N_0, N_{1a}, can not exceed 8·4% with a confidence of 95%. There is considerable disagreement in world literature concerning the incidence of positive scans. Galasko [12, 13] in a 1975 publication described 24% positive bone scan results from a series of 50 patients with breast carcinoma stages T_1, T_2, T_3 (2 patients with large breasts), N_0, N_1.

Ten of these 12 patients died within five years from effects of the carcinoma.

Citrin [14] reported on a series of 83 patients with "primary breast cancer" from which he found 22 (27%) suspected to have bone metastases. In only a few cases metastases were proven.

Green [15] studied a series of 71 breast cancer patients in stages T_1, T_2, N_0, N_1, of whom 7 had positive scans. After one year 3 of them had definite bone metastases. He considered a percentage of 4% proven positive bone scans too small to justify routine skeletal scintigraphy in all patients suspected to have breast cancer.

Sauer [16] included all stages of breast carcinoma in his group of 127 patients and found 12% to have positive bone scans. There was no definite confirmation of the positive scan findings.

Charkes [17] detected a 9% positive scan rate in his series of 34 patients which included all stages of breast carcinoma. In the follow up of these patients all lesions were proven to be caused by metastases.

We consider the high percentages of positive scans found by some workers might be due to an other selection of patients entering their investigations.

Considering our results it would appear that routine pre-operative skeletal scintigraphy is a superfluous examination in patients clinically suspected to have a breast carcinoma stages T_1, T_2, N_0, N_{1a}.

Certainly there appears to be no indication to do routine skeletal X-ray surveys in these groups.

We therefore decided to omit routine bone scanning in patients with breast carcinoma stages T_1, T_2, N_0, N_{1a}. In the small group of patients with more advanced, but still operable breast cancer, we think the study should be continued.

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Kinetic Response of an *In Vitro* "Tumour-Model" (V 79 Spheroids) to 42°C Hyperthermia

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Abstract—*The proliferative changes in multicellular spheroids of Chinese hamster lung cells were investigated under conditions of hyperthermia which lead in combination with radiation treatment to a TER (thermal enhancement ratio) of* 1·63 *By fractionated trypsinization single cell suspensions from successive shells of spheroids were prepared. Cells from the outer region (cycling cells) were found to be partially synchronized by heat and killed in S-phase whereas cells from the center of spheroids (non-cycling cells) became actively proliferating after heat treatment.*

INTRODUCTION

THE APPLICATION of hyperthermia for the treatment of cancer, especially in combination with radiation therapy, is presently under discussion as an alternative to high LET irradiations. There is abundant evidence that cancer cells grown *in vivo* and *in vitro*, are sensitive to elevated temperatures (review: [1]) and that the efficiency of cell inactivation is cell cycle dependent [2–4]. Most of the quantitative data on the cytotoxic effect of heat have been obtained from studies on monolayer cell cultures leaving some uncertainty as to their applicability to the *in vivo* situation. Multicellular spheroids grown from single cell suspensions as first described by Sutherland and co-workers [5] provide a more realistic test system simulating conditions of nodular carcinomas in that cell cycle redistributions and hypoxia occur as they increase in size [6, 7]. These properties make spheroids especially well suited for kinetic studies since both cycling and noncycling cells can be examined concurrently in three-dimensional contact. The kinetic response of exponentially growing V 79 monolayer cultures after hyperthermic treatment has been analyzed in a recent publication [8]. In view of the importance of cell-kinetic alterations for the hyperthermic treatment of cancer we now describe the kinetic response of V 79 spheroid cells after 4 hours at 42°C.

Accepted 16 August 1976.

MATERIAL AND METHODS

Cell culture

Spheroids of Chinese hamster lung cells (line V 79, obtained from Dr. R. B. Painter, San Francisco) were grown to a diameter of 250 ± 20 μm in 100 ml spinner flasks using Eagle's basal medium supplemented with 5% foetal calf serum (Gibco Biocult, BCL 005a) and neomycin sulfate (0·1 g/l). A more detailed description of the culturing technique applied has been published earlier [9].

Fractionated trypsinization of spheroids

The regularly-shaped spheroids offer the unique advantage of removing shell by shell simply by exposing them to a dilute (0·05%) trypsin solution at room temperature. By means of a stereo microscope (magnification 40-fold) with zoom optics this successive detachment of cells from the 250 μm spheroid can easily be followed: During about 6 min cells from a shell of about 20 μm thickness became detached from the spheroids. After stopping the trypsin action by adding an equal amount of medium to the sample the resulting single cell suspension was decanted. This procedure was repeated until the trypsin action had loosened all inner

cells resulting in a complete break up of the spheroid core. Normally, four fractions of single cells representing successive shells of the spheroids could reproducibly be obtained.

Heat treatment and irradiation

Heat treatment was carried out by placing the culture flasks into a waterbath of 42°C whose temperature was kept constant within ± 0·1 °C. After different periods of heat exposure (up to 9 hr) spheroids were subjected to fractionated trypsinization as described above. Only cells of the outer and inner shell, representing cell populations in different state of growth, were tested for survival and prepared for analysis by flow-microfluorometry (FMF).

In another set of experiments spheroids exposed to 42°C for 4 hr were reincubated at 37°C for defined periods of time before trypsinization and preparation for FMF-analysis. Irradiations were performed at 37°C or at 42°C in a ^{60}Co-γ-source at a dose rate of 22 krad/hr as determined by ferrous sulfate dosimetry. Whole spheroids or spheroids trypsinized into single cells were irradiated in small plastic vessels under aerobic conditions and plated as single cells for survival assay. All samples irradiated at 42°C were pretreated with heat as to yield a total hyperthermia of 4 hr.

For survival assay aliquots of single cell suspensions were plated into Falcon tissue culture flasks and incubated for 7 days. Cells yielding colonies with more than 50 cells each were considered as survivors.

Flow-microfluorometry (FMF)

DNA measurements were performed by flow-microfluorometry technique with the "Cytofluorograf" (Model 4801; Bio/Physics Systems) equipped with a 100-channel analyzer. For preparation cells were fixed in 70% ethanol, treated with RNase (0·1% in Tris buffer) for 1 hr at 37°C and subsequently stained with ethidium bromide (0·01 mg/ml) [8]. Under these conditions the amount of dye bound is proportional to the DNA content of the cells [10]. The distribution of fluorescence intensity of the stained nuclear DNA (usually 40,000 cells) was displayed as conventional histogram (cell number vs fluorescence intensity). The percentage of cells in the individual phases of the cell cycle (G_1, S, G_2+M) was calculated from the area under the DNA histograms assuming a rectangular distribution of S-cells between the G_1 and G_2 peak [11]. The reproducibility of independent experiments was better than ± 3%.

RESULTS

The dose–effect curves in Fig. 1 clearly demonstrate the enhanced effectivity for the combination of irradiation with heat exposure compared to irradiation alone. Especially noteworthy is the fact that the second shoulder of the survival at 37°C, indicative of hypoxic cells,

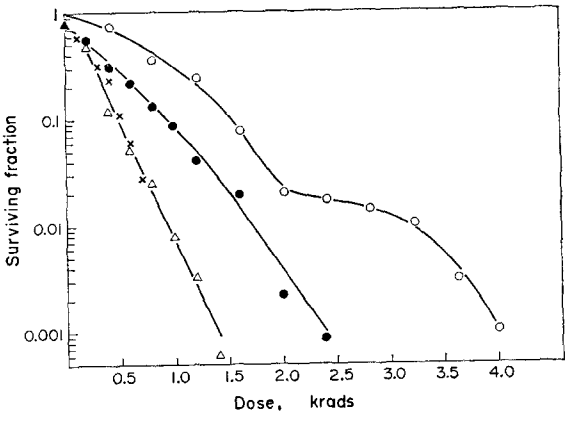

Fig. 1. *Radiation survival curves of V 79 spheroid cells with or without preceding heat treatment. Each point represents the mean from* 3–4 *separate experiments (relative error* ≈ 9·5%).

○ *Spheroids irradiated at* 37°C *and then trypsinized to single cells for survival assay.*

● *Heat treated spheroids were irradiated at* 42°C *and then trypsinized to single cells for survival assay.*

△ *Heat treated spheroids were trypsinized prior to irradiation at* 42°C *and afterwards plated for survival assay.*

× *V* 79 *monolayer cells heat treated* (1 *hr*, 42°C) *prior to irradiation.*

has vanished. The dose–response curves obtained at 42° are related to the net thermal survival of the controls (D = 0) without normalizing these figures to one. Thus, inactivation by heat as well as its radio-sensitizing action can be visualized from Fig. 1. If we define the TER (thermal enhancement ratio) as the ratio of doses at 37°C and at 42°C required to reduce survival to 10% it amounts to 1·63 for whole spheroids. A further depression in survival is obtained when spheroids are trypsinized into single cells prior to irradiation. In this case, the response to hyperthermic irradiation is similar to monolayer cultures irradiated after 1-hr exposure to 42°C (Schlag and Lücke-Huhle, unpublished results).

Figure 2 shows cell cycle distributions of untreated control spheroids trypsinized into four fractions of single cell suspensions (see methods: fractionated trypsinization). Evidently, the percentage of S-phase cells decreases while the number of G_1-like cells increases indicating cells to become pro-

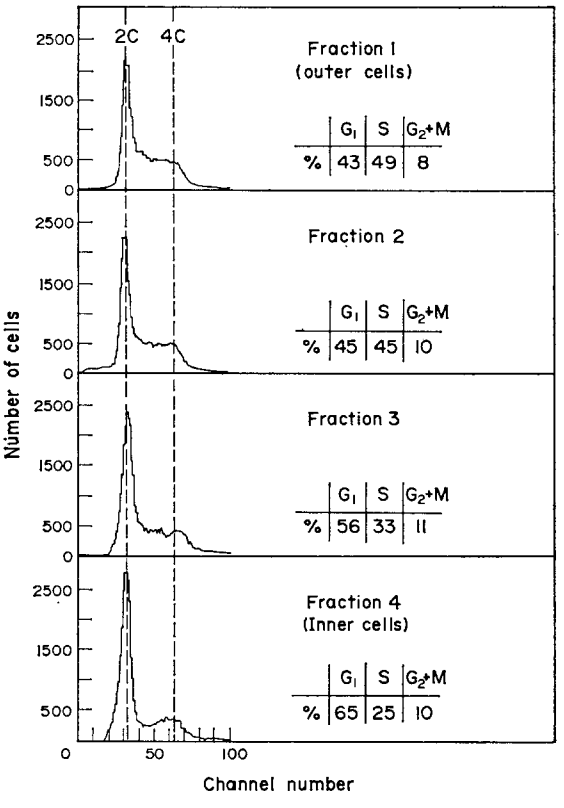

Fig. 2. DNA distribution (shown as analog plots of the multichannel analyzer memory content) of control spheroids obtained by stepwise trypsinization into four successive single cell fractions.

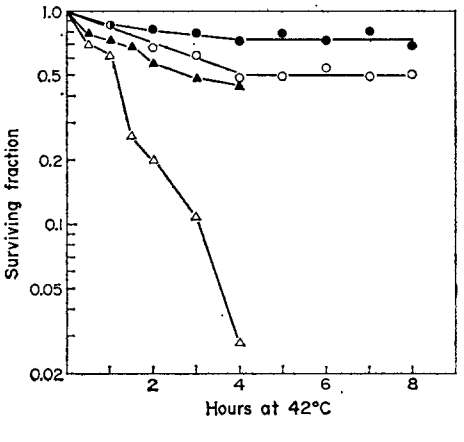

Fig. 3. The effect of hyperthermia at 42°C on monolayer- and spheroid cells in different states of growth. Heat treated spheroids were subjected to fractionated trypsinization and cells of the outer and inner fraction were plated for survival. Each point represents the mean from 3 separate experiments (relative error ≈ 13%).

○ *Outer exponentially growing spheroid cells*
● *Inner cells of spheroids*
△ *Exponentially growing and* ▲ *plateauphase monolayer of the same cell line for comparison (Schlag and Lücke-Huhle,* 1976).

gressively noncycling with increasing depth in the spheroid.

Figure 3 shows the decrease in survival of cells originating from the outer and inner fraction of spheroids after heat treatment at 42°C for different periods of time. Though the inner cells are more resistant to heat exposure both survival curves exhibit a plateau at 50% and 74% survival level, respectively. A comparison with the heat treated monolayer of the same cell line either in exponential or in the plateau phase of growth [8] clearly demonstrates a greater resistance for the spheroid system. Hyperthermia applied for 4 hr leads to small changes only in the fractions G_1, S and G_2+M if cells from totally trypsinized spheroids are analyzed by flow-microfluorometry (Figs. 4a and 4b). With the fractionated trypsinization procedure a more specific investigation of proliferative changes in spheroids is possible as demonstrated by the DNA-distributions in Figs. 4c and 4d. The quantitative difference between the two results can be explained by the presence of a great number of noncycling (G_1-like) cells in theDNA histograms of Figs. 4a and 4b.

Alterations of cell cycle distributions *during* heat exposure are shown in more detail in Fig. 5. The percentages of the individual phases (G_1, S and G_2+M) of outer and inner spheroid cells are plotted against the time of

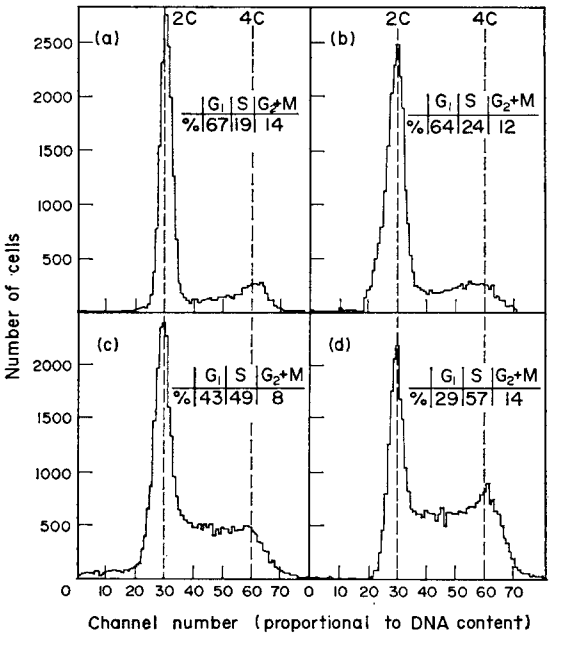

Fig. 4. DNA distributions of spheroid cells before and after heat treatment.

(a+b) Totally trypsinized spheroids.
(c+d) Outer cells of spheroids separated by the fractionated trypsinization procedure.

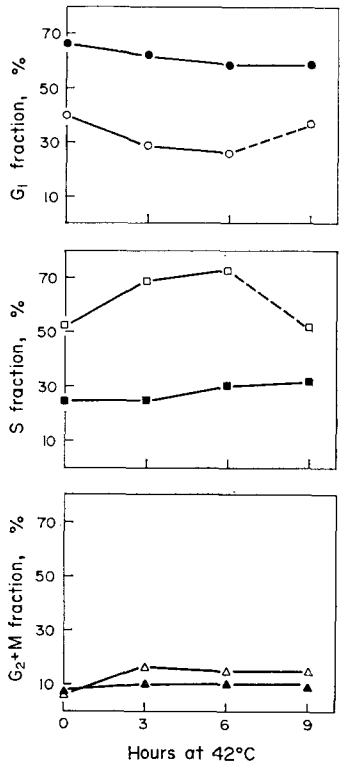

Fig. 5. Percentages of outer and inner spheroid cells in G_1, S and $G_5 + M$ phases as a function of time at 42°C.

Open symbols: outer spheroid cells
Closed symbols: inner spheroid cells.

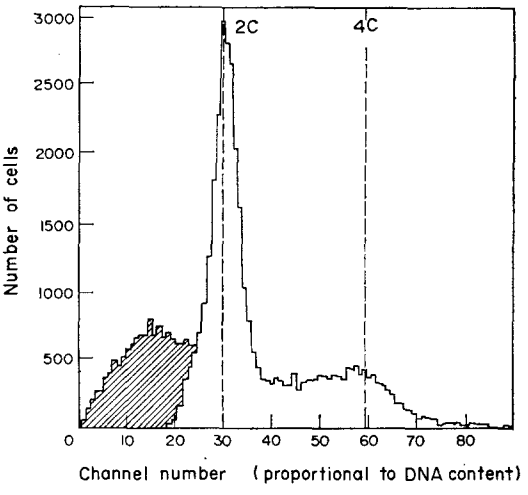

Fig. 6. DNA-histogram of outer spheroid cells after 9 hours hyperthermia at 42°C. Hatched area: cell debris originating from severely damaged cells.

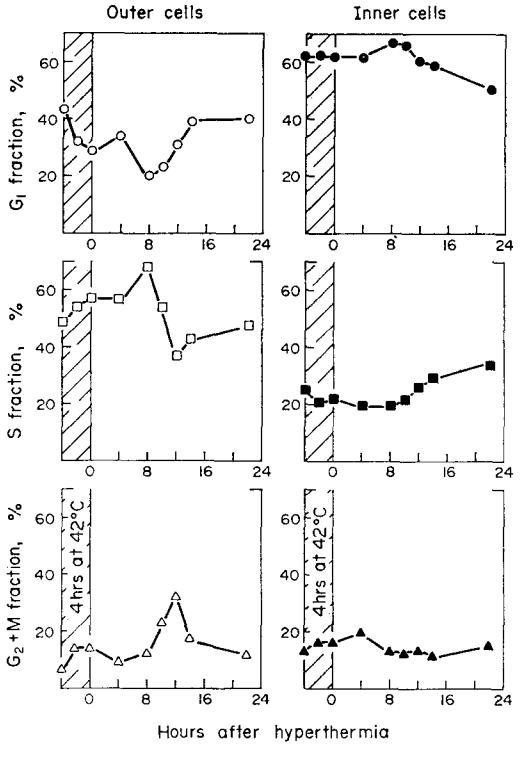

Fig. 7. Percentages of outer and inner spheroid cells in G_1, S and $G_2 + M$ phases as a function of time after hyperthermic treatment.

treatment. While the inner cells show only minor proliferative changes, significant effects are observed within the outer fraction. During heat exposure we find a depletion of G_1-cells indicating a block in $G_2 + M$. The accumulation of cells in $G_2 + M$ is only moderate because cells are simultaneously arrested in S-phase as evident from the increasing level of S-phase cells in the outer exponentially growing spheroid fraction. The sudden decrease of cells in S-phase after 9 hours is not due to a real proliferative event but rather to a staining artifact: Dead S-phase cells being at that time severely damaged appear no longer at the normal position in the cytofluorogram corresponding to their DNA-content but instead as debris on the left hand side of the G_1-peak (Fig. 6).

In Fig. 7 the percentages of G_1, S and $G_2 + M$ cells are plotted against time following a 4 hr heat treatment. The proliferation kinetics of the outer cells after hyperthermia is characterized by the following steps: the $G_2 + M$ block is immediately released followed by the release of the S-block 8 hr later. This generates a wave of synchronous cells which reach maximal values in $G_2 + M$ 12 hr after heat treatment. Fourteen hours after hyperthermia the G_1-compartment is repopulated and after 20 hr the cell cycle distribution approximates that of the control. With the inner cells the situation is different. Four hours after the return to 37°C we find a steady increase of S-phase cells whereas the G_1-fraction decreases.

DISCUSSION

Multicellular spheroids enabled us to study various proliferative effects of heat in a three-dimensional cell culture system without the necessity of generating conditions such as hypoxia or plateau growth in an artificial way. Since the TER of 1·63 as calculated for the spheroids from Fig. 1 comprises both heat-induced cytotoxicity and radiosensitization it is tempting to estimate the fractional effect of pure radiosensitization. This can be done by calculating the ratio of doses at 37°C and 42°C required to reduce survival to 10% of the zero-dose figure. This quantity amounts to 1·46 indicating that under our conditions the effectivity of a combined heat- and radiation treatment is determined mostly by thermal radiosensitization.

The obvious difference in radio-sensitivity between spheroids and single cells after identical preceding heat treatment (Fig. 1) must be ascribed to the phenomenon of extensive intercellular cooperation within three-dimensional cell clones. This contact phenomenon has been shown to render the proliferative cells from the outer spheroid shell more resistant to γ-irradiation at 37°C as compared to exponentially growing monolayers [9]. Recent irradiation experiments with negative pions revealed that the spheroid cells exhibit contact resistance even with respect to radiation of higher LET [12]. From Fig. 3 it is evident that spheroid cells can tolerate more hyperthermic damage: although the outer spheroid cells have the same cycle distribution as the exponentially growing monolayer they are exceptionally thermoresistant. Similarly, the inner spheroid cells are more resistant to heat than the plateau phase monolayers. In addition, Fig. 3 shows that cycling cells are more sensitive to heat than noncycling cells. The plateau in the spheroid survival curves indicates that, after some initial cell killing, the surviving cells are perfectly protected by intercellular contact against further heat damage. Since the final rate of inactivation compares fairly well with the frequency of S-phase cells we may conclude that spheroid cells in S-phase are most heat sensitive and therefore most efficiently killed by heat. This view is supported by the results of the proliferation kinetic studies during heat exposure. From Fig. 4 it can be seen that part of the cells accumulated in S-phase during hyperthermia leave that phase as cell debris (Fig. 6) suggesting lethal damage to these cells. Pronounced heat sensitivity of S-phase cells was also observed for monolayers of V 79 cells heated both in logarithmic and plateau phase [8] and is in agreement with the results by other authors [3, 4].

Elimination of hypoxic cells is evident from the lack of the hypoxic shoulder in the survival curve of spheroids irradiated after heat treatment. However, our results do not allow a distinct conclusion as to the process leading to this elimination. Although reoxygenation of the inner cells due to killing of the outer cells cannot be completely ruled out we prefer the explanation based on the results by Schulman and Hall [13] according to which hypoxic V 79 cells are more sensitive than aerobic cells. As only a few per cent of the inner spheroid cells are hypoxic their preferential inactivation by heat would not contradict the interpretation offered for the curves in Fig. 3 (see above). Synchronization of the outer exponentially growing spheroid cells is evident from Fig. 7. The pattern of synchronization is similar to that observed after heat treatment of exponentially growing V 79 monolayers [8] and agrees with the findings described for other cell lines [14]. In contrast to the outer cell fraction the G_1-like inner cells of spheroids do not participate appreciably in synchronous growth, the kinetic changes being rather moderate during and after heat exposure. Therefore, we may assume that a large portion if not all of the inner G_1-like cells reside in a resting (G_0)-phase which is not resolvable by the staining technique applied. The steady increase of S-phase cells with the concomittant decrease of the G_1-like cells, however, indicates a progressive transition of the inner cells into the proliferative state. Clearly, this is the consequence of renutrition and reoxygenation of the noncycling cells due to killing of the outer cells by heat treatment. Kal and Hahn [15] presented evidence for a recruitment of noncycling cells after combined heat- and X-ray treatment of EMT-6 tumour by applying a mathematical model. But so far recruitment of this type was not shown directly and only the method of fractionated trypsinization offered the possibility to follow the kinetic response of cycling *and noncycling cells* in a three-dimensional system.

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The Pars Intermedia and Renal Carcinogenesis in Hamsters*

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Abstract—*Male hamsters, 3 months of age, were treated for 9 months by thrice weekly injections of diethylstilboestrol (DES). All animals developed kidney tumours and histopathological examination showed that the intermediate lobes of the pituitary were hyperplastic and neoplastic. The content of melanocyte-stimulating hormone (MSH) in the pituitary glands of 17 control and 12 DES-treated animals was measured by bioassay and radioimmunoassay. Whan compared with control pituitaries, the concentration of immunoreactive a-MSH in treated animals was significantly elevated* ($P < 0.005$) *with significant positive correlation between pituitary weight and total a-MSH content. The levels of bioactive MSH were also raised. Serum levels of immunoreactive a-MSH were consistently higher in treated animals.*

The possibility exists that the induction of kidney tumours by oestrogens in the male hamster is mediated via the pituitary gland and that MSH may be of significance in the process of carcinogenesis.

INTRODUCTION

THE METHOD by which oestrogens induce renal tumours in the male hamster is uncertain. Kirkman [1] considered the possibility that such hormones acted directly on the kidney since stilboestrol implanted in the spleen and, therefore, in the path of hepatic drainage failed to induce renal tumours. Algard [2] supported this view since he found that organ cultures of renal tumours were oestrogen dependent.

Another suggestion was that oestrogen-induced tumours resulted from changes in the pituitary gland. Long-term oestrogen treatment has been shown to cause a significant increase in the number of prolactin-secreting cells while a prolactin-inhibiting drug (CB154), when given in combination with stilboestrol, reduced the incidence and severity of kidney tumours in the hamster [3]. Oestrogen treatment also has been found to induce hyperplastic and neoplastic change in the intermediate lobe of the pituitary gland of the hamster [3–5].

Melanocyte-stimulating hormone (MSH) is produced in the intermediate lobe of the pituitary [6] and, in the present study, the possibility has been examined that increased amounts of MSH are produced by the enlarged intermediate lobe resulting from prolonged oestrogen treatment.

MATERIAL AND METHODS

Fifty-four male Syrian golden hamsters (*Mesocricetus auratus*), aged 3 months at the beginning of the experiment, were used. The animals were housed individually in a light- and temperature-controlled room, weighed at weekly intervals and maintained on dog-chow and tap water *ad libitum*, supplemented twice weekly with cabbage. The hamsters were divided randomly into two groups, each of 27 animals. One group consisted of untreated controls while the animals of the second group were treated with diethylstilboestrol (DES). Each animal received one s.c. injection of 0·6 mg DES (Sigma) suspended in 0·2 ml distilled water three times per week. After a total dose of 65 mg DES over a period of 9 months, the animals were finally weighed, killed by cervical dislocation and autopsied.

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The pituitaries of 17 control and of 12 DES-treated hamsters were removed immediately, weighed and placed in separate phials containing 1 ml of 0·25% glacial acetic acid and stored at −20°C for MSH assay. Pituitary glands from the remaining treated and control animals were weighed, fixed in 10% formal-sublimate and stained by Herlant's tetrachrome method for cytological examination. At post-mortem, testes and kidneys were weighed and fixed in 10% formal-saline together with representative portions of liver, spleen, lungs and brain. The tissues were embedded in paraffin-wax, sectioned at 5 μm and stained with haematoxylin and eosin for histopathological examination.

MSH assays

The method of Howe and Thody [7] was used for the bioassay of total MSH activity in extracts of pituitary tissue. A radioimmunoassay [8, 9] was used for the measurement of pituitary and serum a-MSH levels.

Statistical analysis was done by the 2-tailed Student's *t*-test using a Data General Corporation Nova 820 computer programmed to correct for inter-group variance difference when computing *P* values.

RESULTS

Of the 27 DES-treated animals, 3 died early in the course of the experiment and were discarded. In the remaining animals, statistical analysis failed to show any significant difference in body weight between treated and control animals ($P > 0·05$) (Table 1) and, consequently, it was possible to make direct comparisons of organ weights. The kidneys of the DES-treated animals were significantly heavier than those of controls ($P < 0·0005$) (Table 1). This difference in weight was clearly associated with the presence of tumour tissue in the kidneys of all treated animals. In general, kidneys were enlarged, irregular in outline with protruding tumour nodules and showed multiple neoplastic foci of varying size—3 mm to 2 cm in diameter—that were mainly bilateral and cortical in distribution. Lesions were solid, cream coloured or, often, consisted of haemorrhagic cysts intermixed with solid tumour. Central necrosis was prominent in the larger lesions. Four of the animals had haemorrhagic ascites with metastatic deposits on the serosal surfaces of abdominal organs. Histopathologically, a gradation of change from increased basophilia and hyperplasia of tubular epithelial cells to frank neoplasia could be observed within any one kidney. Because of origin from tubular epithelium and their invasive tendencies, the tumours were classified as carcinomas.

Table 1. *Effect of DES treatment on weight* (g) of body, kidneys and pituitary in male hamsters*

Weight	Control(27)†	DES(24)	
Body	133 ± 2	138 ± 3	N.S.D.‡
Kidneys	0·5199 ± 0·0106	1·5176 ± 0·2464	< 0·0005§
Pituitary	0·0032 ± 0·0002	0·0369 ± 0·0116	< 0·005

*Expressed as the mean weight for the group ± S.E.M.
†The number of animals in the group.
‡No significant difference between groups.
§The value of *P*, denoting the significance of difference between groups.

The pituitary glands from DES-treated animals were larger and significantly heavier than those from control animals ($P < 0·005$) (Table 1). The anterior lobe was compressed to a thin shell of tissue covering a bulging intermediate lobe. The main alteration lay in the cellular composition of the adenohypophysis with a significant increase in the number of prolactin-secreting cells and a decrease in the number of somatotrophin-secreting cells and in the basophils. Histopathological examination showed that the considerable enlargement of the pituitary was mainly restricted to the intermediate lobe. The cells were hypertrophic and hyperplastic with frequent evidence of mitosis. Neoplastic change had supervened and infiltration of the posterior lobe and infundibular stalk by cells of the intermedia was common with, in some cases, almost total replacement of the posterior lobe. Occasionally, invasion of the anterior lobe was in evidence. Those findings confirm our previous results [3].

The results of the MSH assays are shown in Table 2 and Fig. 1 and 2. The mean level of immunoreactive a-MSH in the pituitary glands of the 17 control animals was 471 ± 50 ng/pituitary. By bioassay, the mean MSH activity in 5 of those pituitaries was equivalent to 3980 ± 370 ng a-MSH/pituitary. Thus a-MSH accounted for approximately 10% of the total bioassayable MSH in the hamster pituitary.

There was a significant increase ($P < 0·005$) in the mean level of immunoreactive a-MSH (16,100 ± 430 ng/pituitary; 386 ± 69 ng/mg pituitary) in the pituitaries of DES-treated animals when compared with controls (Fig. 1). A significant positive correlation was found

*Table 2. Effect of DES treatment on α-MSH level * in male hamsters*

	Controls	DES
Pituitary MSH		
No. of assays	17	12
Total content (ng/gland)	471 ± 50	14,810 ± 4167
	$P < 0{\cdot}005$†	
Concentration (ng/mg wet wt.)	146 ± 14	360 ± 69
	$P < 0{\cdot}005$	
Serum MSH		
No. of assays	4	10
Concentration (pg/ml)	453 ± 152	3190 ± 521

*Expressed as mean ± S.E.M.
†The value of *P*, denoting the significance of difference between groups.

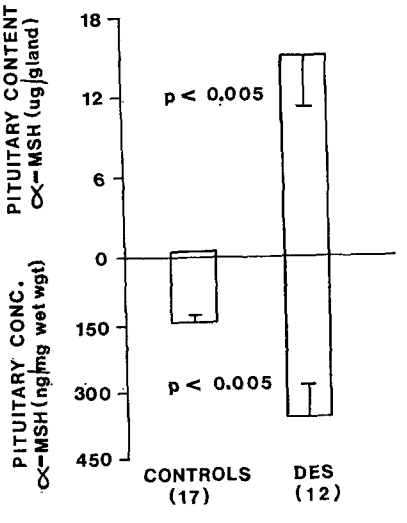

Fig. 1. Effect of DES treatment on mean pituitary levels of α-MSH in male hamsters. The vertical bars represent S.E.M. and the numerals in brackets indicate the number of animals examined. The values of P *denote the significance of difference between groups, calculated by two-tailed Student's* t*-test.*

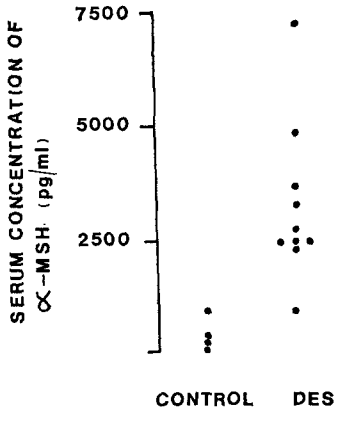

Fig. 2. Effect of DES treatment on serum levels of α-MSH in male hamsters.

between pituitary weight and total content of a-MSH ($r = 0{\cdot}6$; $P < 0{\cdot}02$). Three pituitaries from DES-treated animals bioassayed for MSH were found to contain markedly increased activity (equivalent to 119,260 ± 47,840 ng α-MSH/pituitary). The immunoreactive/bioactive ratio in the pituitaries of 2 of those animals was higher than normal and suggested a preferential increase in the α-MSH content of the glands.

To measure serum levels of immunoreactive α-MSH it was necessary to pool sera from several animals so that ultimately there were insufficient numbers for statistical analysis. However, the values illustrated in Fig. 2 indicate that serum α-MSH levels were consistently higher in DES-treated than in control animals.

There have been several reports that prolonged oestrogen treatment of the hamster leads to hyperplastic and neoplastic changes in the intermediate lobe of the pituitary [3–5]. The present results confirm those findings but also show that the enlarged intermediate lobes are capable of producing and releasing increased amounts of MSH.

Various forms of MSH exist and, in the normal hamster, Penny and Thody (unpublished data) have estimated that α-MSH accounts for approximately 10% of the total bioassayable MSH of the pituitary. That figure was confirmed in the present study. However, in 2 of the 3 DES-treated hamsters, the pituitaries of which had also been bioassayed, a greater proportion of α-MSH was found indicating that the hyperplastic and neoplastic intermediate lobes were preferentially producing α-MSH. That may have been a common feature of all the enlarged lobes since most of them contained exceedingly high levels of immunoreactive α-MSH.

Hamilton *et al.* [3] have reported that a prolactin inhibitor (CB154), when given concurrently with stilboestrol, reduced the incidence and severity of kidney tumours. The effect of CB154 may be related to interference with prolactin secretion although Hamilton *et al.* (unpublished data) failed to induce renal tumours with prolonged prolactin treatment and was unable to demonstrate that, when given concurrently with DES, prolactin influenced the induction or severity of the kidney tumours. However, Penny and Thody (unpublished data) have found recently that, in the rat, CB154 inhibits the secretion of MSH and that finding suggests the possibility of a relationship between renal carcinogenesis and MSH secretion. There is emerging evidence that indicates that MSH acts on the kidney.

Orias and McCann [10] reported that α-MSH and β-MSH induced natriuresis in the rat while increased levels of immunoreactive β-MSH have been found in patients with chronic renal failure [11, 12]. Smith and Shuster (personal communication) consider that, in man, the kidney may metabolise MSH-like peptides. Such metabolites may occur in the hamster and under conditions of increased MSH secretion may lead to neoplastic change in the kidney. That possibility must now be examined.

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The Effect of BCG on *In Vitro* Immune Reactivity and Clinical Course in Dogs Treated Surgically for Osteosarcoma*

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Abstract—*Cell mediated reactivity (CMR) and serum blocking activity (SBA) were assessed postsurgically in* 11 *dogs treated surgically for osteosarcoma. Six dogs received Bacillus Calmette-Guérin (BCG) and* 5 *served as controls. SBA decreased significantly soon after surgery in both groups. Two to three months after surgery SBA was slightly higher in the controls than in the BCG group. However, the postsurgical SBA in BCG recipients rose to about the same level as in the controls at* 4–12 *months, correlating in time with radiographic appearance of metastasis. This suggested that BCG delayed SBA for several months.*

The median survival time of the BCG treated dogs was 40 *weeks, significantly longer than the* 13 *week median survival time in the control group. The cumulative life table method for estimation of survival rate failed to show an effect of BCG on survival.*

INTRODUCTION

BACILLE CALMETTE-GUÉRIN (BCG) is a non-specific immunestimulant, which enhances the immune response to a wide variety of antigens including tumor specific antigens [1–3]. It can be used either locally or systemically. Several investigators have demonstrated that BCG vaccine given before or after tumor grafting in a syngeneic murine host can retard subsequent tumor growth [4–7]. The suppressive effect of BCG on tumor growth can be most readily demonstrated when the tumor has antigens differing from those of the host [4] and when the tumor mass is low [4, 5, 8]. An immunocompetent host is a requisite for the manifestation of BCG effects.

BCG immunotherapy has been utilized for human acute lymphocytic leukemia and malignant melanoma. Host immune responses to tumor specific antigens can be demonstrated in both neoplasms [9, 10].

Mathé *et al.* [11] showed significant prolongation of remission in leukemia patients treated with BCG. Rosenthal *et al.* [12] suggested the value of prior BCG vaccination in preventing leukemia in children. Morten *et al.* [10, 13, 14] and Nathansen [15] showed that BCG injected into the site of cutaneous melanoma retarded distant as well as local lesions while others found regression of only the injected lesions [16, 17].

In a recent study on the use of BCG in the treatment of metastatic melanoma, Prehn *et al.* [18, 19] reported that the *in vitro* lymphocyte cytoxicity in the presence of patient's serum showed correlation with the status of the clinical disease.

The treatment regimens employed have been empirical with regard to dose, type of BCG and route of administration [14–17, 20–27]. Whether BCG will cause inhibition or stimulation of tumor growth when administered *in vivo* is determined by a number of factors, among which the dosage [28] and route of administration, tumor volume at the time of treatment, and status of tumor immunity, appear to be of major importance [29]. The best preparation for treatment of a given tumor, the optimum dose and route of administration

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Table 1. *Clinical data on osteosarcoma patients*

Dog no.	Age (yr)	Weight (kg)	Sex	Breed	Tumor site and duration of clinical signs	Results
1	5	10	FS	Cocker	Femur (D) 4 weeks	†NED, 71 weeks post-op.
2	0·8	33	M	German shepherd dog	Femur (D) 3 weeks	Skin metastasis, 26 weeks post op. *EUT. 8 weeks later with widespread metastasis.
3	1·3	35	M	Old Eng. sheep dog	Femur (D) 3 weeks	EUT. 3 weeks post-op., widespread metastasis.
4	2	18	F	Springer spaniel	Ulna, radius (D) 6 weeks	Prescapular L.N. metastasis noted and excised 12 weeks post-op. Lung metastasis noted 36 weeks post-op. and removed 40 weeks post-op. Multiple lung metastases 46 weeks post-op. EUT. 60 weeks post. op.
5	2	23	F	Irish setter	Femur (P) 6 weeks	NED, 46 weeks post op.
6	7	10	FS	Wire-haired terrier	Humerus (D) 8 weeks	Lung metastasis noted 20 weeks post-op. Developed ‡HOP 4 weeks later and EUT.
7	6·5	81	FS	St. Bernard	Radius (D) 3 weeks	EUT. 6 weeks post-op. Lung metastasis found at post-mortem.
8	2·5	35	M	German shepherd	Tibia (D) 6 weeks	Lung metastasis noted 10 weeks post-op. EUT, 2 weeks later.
9	7·5	58	M	St. Bernard	Humerus (Mid-shaft) 6 months	Lung metastasis noted 8 weeks post-op. Died naturally 4 weeks later.
10	2·5	37	M	Irish setter	Tibia (P) 4 weeks	NED, 24 weeks post-op.
11	9	33	F	Weimar-aner	Radius (D) 2 months	NED, 70 weeks post-op. Tonsillar carcinoma noted and excised 70 weeks post-op. EUT. with widespread lung and bone metastases of carcinoma and osteosarcoma 78 weeks post-op.

*EUT.—Euthanatized
†NED.—No evidence of disease clinically and radiographically
‡HOP.—Hypertrophic pulmonary osteoarthropathy

have only recently undergone clinical investigation [2, 30]. The route of BCG administration may determine whether tumor suppression or enhancement is induced [31] and may influence the severity of complications [32]. Side effects reported include fever, nausea, rash, local skin necrosis, lymphopenia and leukopenia [15].

The aim of this work was to determine the effects of immunotherapy with BCG on dogs treated surgically for osteosarcoma. The effects of BCG on the clinical course of disease were correlated with 2 *in vitro* parameters, cell mediated reactivity (CMR) expressed as the percentage inhibition or stimulation of target cell numbers in an average of 6 replicates and serum blocking activity (SBA) expressed as the percentage change in the number of target cells in the presence of patient's lymphocytes and serum compared to normal serum in an average of 6 replicates.

MATERIAL AND METHODS

Animals bearing tumors

Eleven dogs with histologically diagnosed osteosarcoma and without clinical or radiographic evidence of metastasis were studied (Table 1). All were patients at the Small Animal Hospital, University of Pennsylvania, School of Veterinary Medicine. Amputation of the tumor bearing limb was performed at the time of initial diagnosis. Post-operatively dogs were immunologically tested and examined clinically and radiographically at monthly intervals whenever possible. Complete blood counts were performed at each examination.

Eleven dogs were selected sequentially

between May 1973 and September 1974 from patients seen at our hospital. The first 6 of these dogs were given BCG* and the last 5 served as controls. A standard dose of 1 ml of $1{\cdot}5+10^7$ viable organisms was injected intradermally in the flank area in each of the six dogs given BCG. The total dose was divided into 0·20 ml. portions administered at five separate sites. The first dose was administered 9 days after surgery. The BCG treatment was repeated at 2-week intervals for one year.

Tumor growth stimulation-inhibition test

The microcytotoxicity assay, colony inhibition test, described by Hellstrom [33], and modified by Fidler for the tumor growth stimulation-inhibition test [34] was used as a routine procedure. This modification increased the plating efficiency of tumor cells which improved the accuracy of initial lymphocyte to target cell ratios. This test has been described in detail [34–36]. Briefly, tumor tissue was obtained aseptically at surgery and prepared in the laboratory within 30 minutes. The tumor was minced, trypsinized and resuspended in complete Eagle's minimum essential medium (CMEM). The tumor cells were then plated after being passed through a gauze covered funnel to give a single tumor cell suspension. Viable, plastic adherent cells were harvested after 12 hr. After viability tests, the number of live target cells was adjusted to 30,000/ml media. Tumor cell preparations were incubated in CMEM; 50% CMEM and 50% autologous serum; 50% CMEM and 50% normal dog serum for 15 min. Fibroblasts from skin biopsy (dog 6) were prepared as described for tumor cells.

White blood cells were obtained by sedimenting heparinized blood on dextran-citrate (1·5 ml of 2·4% sodium citrate and 1·5 ml of 10% dextran 250,000 M.W., mixed with 7 ml of heparinized blood) [37]. After sedimentation, the cell suspension was passed through a glass-wool column to remove adherent cells, washed and diluted. Viability was determined by trypan blue exclusion and exceeded 95% in all cases. The yield of non-glass adherent, mononuclear cells (lymphocytes) was approximately 35% using this method.

Tumor cells and lymphocytes were combined to yield target cell to lymphocyte ratios of 1:100, 1:1000 and 1:10,000. After rotation for 90 min. to enhance cell to cell interaction the tumor-lymphocyte mixtures were placed in Falcon Microtest I plates and incubated at 37°C in 5% CO_2. Six wells per test group per assay day (2 and 5) were filled. Each well contained 30 tumor cells and varying numbers of lymphocytes in a 0·02 ml suspension. To achieve accurate cell dispensing, a sterile disposable 1 ml tuberculine syringe inserted into a Hamilton repeating dispenser was utilized (Hamilton Co., Nevada, USA). Plates were harvested and stained on days 2 and 5.

Analysis of CMR and SBA

The *in vitro* CMR response of all patients to their tumors was measured sequentially. Serum blocking activity (SBA) and cell mediated reactivity (CMR) were determined at surgery, at monthly intervals after the first BCG injection and either at post mortem or 12 months after initiation of treatment. In the measurement of SBA all sera collected during the observation period were evaluated simultaneously and compared to presurgical serum used as a base line reference.

Standard statistical procedures were used to evaluate results. Survival time refers to the interval between excision of the primary tumor and euthanasia (9 dogs) or natural death (2 dogs). Inasmuch as 9 dogs were euthanatized at different stages of their terminal disease (dog 7 before developing terminal osteosarcoma), the survival times are biased toward a minimum in contrast to the situation in humans where death occurs naturally.

Lymphocyte count refers to the absolute number of lymphocytes from differential blood count.

RESULTS

CMR and SBA levels

The number of dogs with presurgical CMR inhibition in the BCG group were not significantly different from those in the control group, $\chi^2 = 0{\cdot}736$, $d.f. = 1$, $P > 0{\cdot}30$. Therefore BCG and CMR were treated as independent variables.

No consistent relationship between CMR and SBA and clinical metastasis was found during the follow up period in either the BCG treated dogs or the controls.

Experiments were done comparing our CMR results with lymphocytes from tumor hosts to lymphocytes from normal dogs. The addition of normal lymphocyte preparations may have some non-specific effect of growth of the target cells [38–42]. The effect of fibroblasts was tested using normal dog lymphocytes from different donors. The significance of these

*Obtained from Trudeau Institute, Saranac Lake, N.Y.

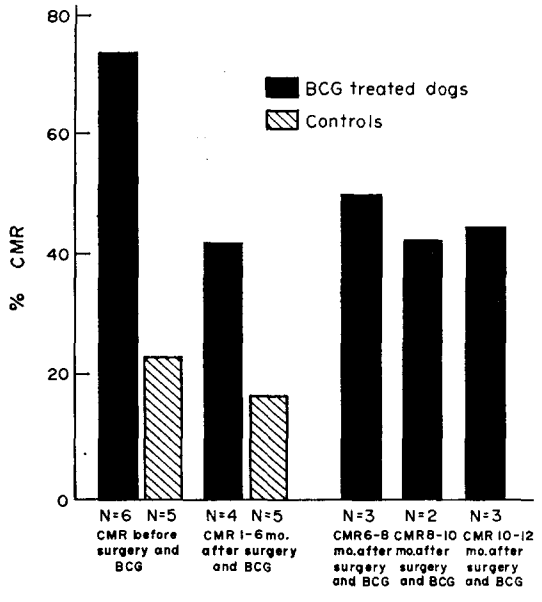

Fig. 1. CMR = Cell mediated reactivity, N = Number of dogs tested at each time interval, BCG = Bacillus Calmette-Guérin.

experiments was to demonstrate that lymphocytes from normal individuals may occasionally react against malignant and normal cells in a non-tumor specific fashion. Normal fibroblasts were destroyed by normal lymphocytes in two out of seven experiments.

Removal of the primary tumor was associated with a 93% decrease in the SBA. The decrease in CMR was not statistically significant even though the means dropped from 73 to 40% during the first six months.

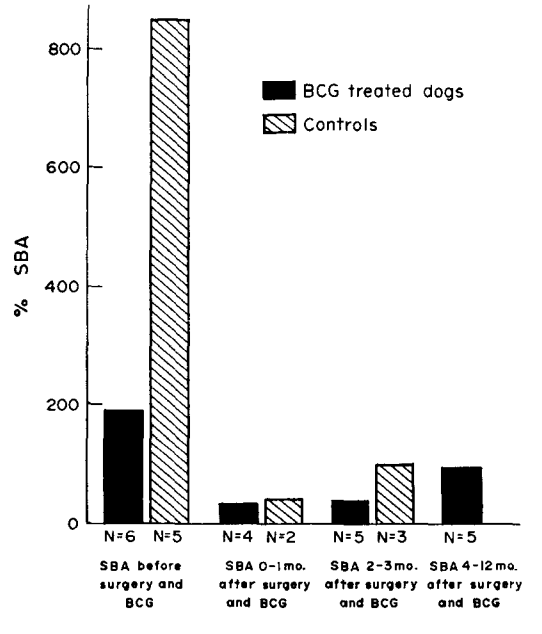

Fig. 2. SBA = Serum blocking activity, N = Number of dogs tested at each time interval, BCG = Bacillus Calmette-Guérin.

Figures 1 and 2 illustrate the sequential means of CMR and SBA in BCG treated and control groups 1–12 months after surgery. SBA values show significant decreases from presurgical levels in test and control groups to low levels 1 month after surgery. At 2–3 months after surgery the SBA levels were slightly higher (statistically insignificant) in the control group than in the BCG group. However, SBA in BCG treated dogs rose to about the same level as in controls 4–12 months after surgery.

Radiographic evidence of metastatic disease was present in 1 of 6 BCG dogs 2–3 months postsurgically and in 4 of 6 dogs, 4–12 months after surgery. Figure 3 illustrates the changes in SBA in the combined BCG and control groups.

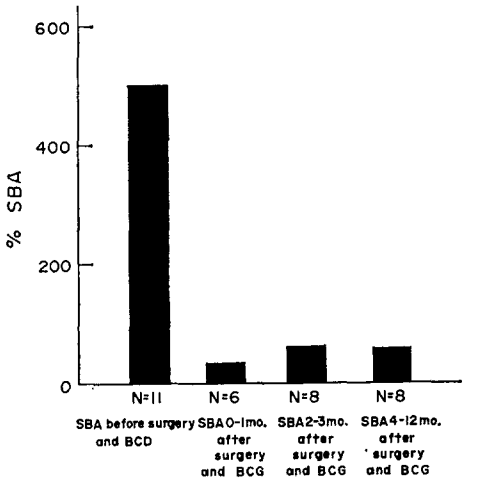

Fig. 3. SBA = Serum blocking activity, N = Number of dogs tested at each time interval, BCG = Bacillus Calmette-Guérin.

Effects of BCG on lymphocyte counts

Although the mean postsurgical lymphocyte counts in the 6 BCG dogs increased 45·5% above the presurgical values (mean went from 2128 at surgery to 3096 at the end of the experiment), this gradual rise in the numbers of lymphocytes was not significant when evaluated by least squares regression analysis ($b = 0{\cdot}794$, $d.f. = 38$, $P > 0{\cdot}05$).

The median absolute lymphocyte count in the presurgical period was 1830 (6 BCG treated and 5 control dogs) while in the dogs with terminal disease the corresponding value was 1410. The decrease in absolute numbers of lymphocytes in terminally ill animals was statistically significant, $P < 0{\cdot}05$.

Effects of BCG on survival time

The median survival time of 40 weeks in the BCG group was significantly prolonged when

analyzed by the median test ($\chi^2 = 4.0$, $d.f. = 1$, $P < 0.05$), as contrasted to 13 weeks in the control group.

The life table method was used for estimation of survival rates [43, 44]. Survival rates were calculated for the 6 dogs which received BCG and for 65 "historical controls" which had undergone amputation for osteosarcoma at this institution [45]. Based on these survival rates, 7·8% of the 65 historical controls would be expected to survive for 19 months postsurgery in contrast to the 50% of the 6 BCG dogs which

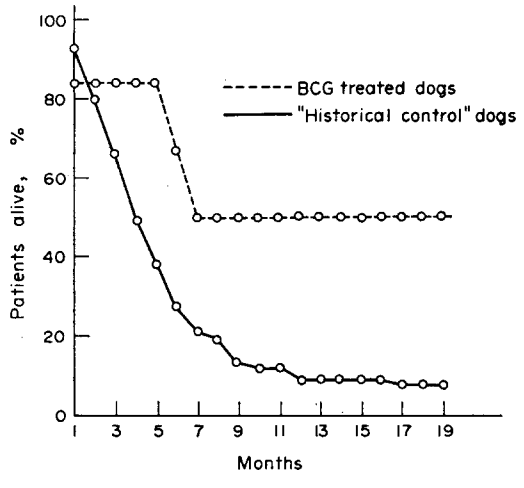

Fig. 4. Proportion of dogs alive vs. the number of dogs at risk at each point in time in 65 historical control dogs and 6 BCG treated dogs respectively.

were alive at that point in time (Fig. 4). However, the 95% confidence limits for the survival rates expressed as percentage of BCG dogs surviving were 10 and 90% while for the "historical controls" they were 0 and 17·6%.

In order to calculate the expected survivors in the BCG group the cumulative probabilities for surviving for different periods of time found in the life table for the "historical controls" were used, Table 2 [46]. A total of 1·692 survivors and 4·308 deaths was expected in the 6 BCG treated dogs. The actual cumulative survival of 3 BCG treated dogs for 19 months was not statistically significant, $\chi^2 = 1.408$, $d.f. = 1$, $P > 0.20$.

Rates of metastasis were also analysed by the life table method [43, 44, 47]. In Fig. 5, the proportion of dogs free of metastasis vs the number of dogs at risk at each point in time was plotted. No significant difference was found between the proportion of BCG treated and control dogs free of metastasis.

Pathologically no gross differences were noted in the number of size of metastases between BCG and control dogs.

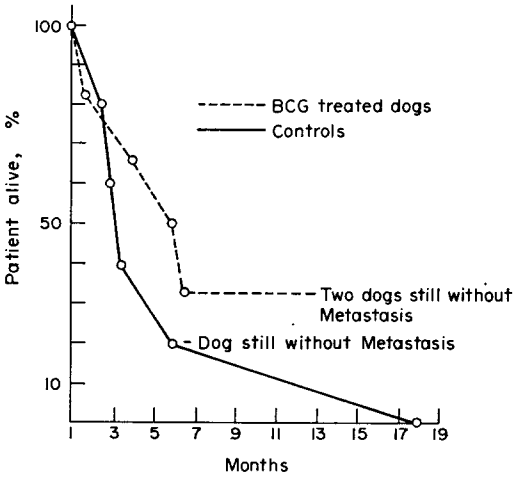

Fig. 5. Proportion of dogs free of metastasis vs. the number of dogs at risk at each point in time in 6 BCG treated and 5 control dogs respectively.

Local effects of BCG

The dogs were reexamined at 2 week intervals. No local changes except for some induration in the skin were noted 28 days after the initial injection of BCG. However, at 42 days after the initiation of BCG therapy, local ulcerations 0·2–1 cm in diameter were noted at the initial sites of injection. Upon subsequent examination 2 weeks later the lesions were healing well. They were completely healed 28 days after initially observed.

Table 2. Expected survival in 6 BCG-treated dogs at 19 months

Observation time (months)	Dogs	Expected survivors	Observed survivors
1	1	1 × 0·939 = 0·939	5
6	1	1 × 0·276 = 0·276	3
7	1	1 × 0·215 = 0·215	3
13	2	2 × 0·092 = 0·184	3
19	1	1 × 0·078 = 0·078	3
	6	Total: 1·692 Survivors expected	

DISCUSSION

These studies examined the possibility of immunostimulatory-inhibitory effects following BCG injection by measuring differences in *in vitro* immunoreactivity before and after surgery and BCG injection. The data were compared with similar data from a control group which did not receive BCG. By only using dogs with surgically extirpated tumors and no radiographic evidence of metastasis, it was hoped to avoid blocking factors which, in some human cancer patients, have been shown to disappear soon after the heavy tumor burden has been removed [48]. By BCG treating only dogs with surgically extirpated tumors it was hoped to minimize the risk of stimulating SBA and tumor growth by BCG.

Stolfi *et al.* [49] found that SBA of tumor bearing mice became undetectable within 15 days after surgery. This gives support to the idea that blocking factors are circulating complexes of tumor antigens, and antibody formed by the host. When a tumor antigen and specific antibody are mixed, the mixture blocks, although neither the antigen nor the specific antibody alone blocks when tested under standard conditions [50]. If, however, the antigen is added to immune lymphocytes, it can block as well [51]. Regarding the class of antibody involved, Jose *et al.* [52] demonstrated blocking activity associated with subclasses IgG_1 and IgG_3, and lesser activity in IgG_4. Most other workers associate them with the IgG_2 fraction [53, 54].

Surgical removal of grossly detectable tumor was associated with decreased SBA, a change that should favor destruction of residual tumor cells. SBA decreased 1 month after surgery, probably due to elimination of tumor antigens from the primary osteosarcoma. It is noteworthy that even in these dogs in which a beneficial shift in immunological reactivity had occurred, tumor metastasis became clinically overt later.

The mechanism by which the immune system responds to BCG is still not clear. Zbar *et al.* [55] postulated the mechanism of intralesionally injected BCG mediated tumor cell death in a guinea pig model in 3 steps: (a) the host produces lymphocytes which recognize antigen of BCG, (b) specifically sensitized lymphocytes react with BCG, leading to the production of soluble mediators of cellular immunity. One of these factors (migration inhibition factor) promotes local accumulation of monocytes essential for tumor cell destruction, and (c) monocytes process tumor antigens leading to the formation of sensitized lymphocytes that recognize the distinctive antigens on the tumor cells.

Snodgrass *et al.* [56] presented histologic evidence suggesting that a non-specific granulomatous reaction, characterized by infiltrating histiocytes was a necessary part of the anti-tumor response in BCG treated animals. Non-specific destruction of tumor cells accompanied a specific reaction of lymphocytes with the sensitizing antigen in close proximity to the neoplastic cells.

Further, Borsos *et al.* [57] verified the presence of common antigens between BCG organisms, line 10 hepatocarcinoma of strain 2 guinea pigs and a human melanoma cell culture. One question has now been raised with regard to BCG immunotherapy by Hanna and Bucana [58]. Does the humoral immune reactivity to common antigens under certain conditions antagonize development of CMR? An early and pronounced humoral immune response to BCG may block cell mediated immunity either on the sensitized lymphocyte or on the tumor cell level. This could depend on the nature of the BCG used for the therapy and the quantitative levels of common antigenic sites on the tumor cells. A specific humoral antibody response to BCG in the dog has not been demonstrated, but probably exists. The general nature of the immunologic cross reactivity between tumors of different histologic types with BCG or other microorganisms needs to be nvestigated.

The significant difference in median survival times of the 6 BCG treated dogs and the 5 control dogs indicates a possible effect of BCG on duration of survival of surgically treated osteosarcoma bearing dogs. As of 1 February, 1976, 2 of the BCG treated dogs and 1 control dog in the present study were still alive and well at 131, 106 and 84 weeks after surgery respectively.

More dogs should be studied to evaluate fully the significance of treatment with the particular strain and dose level of BCG used in this study. Probably due to the small number of dogs tested the cumulative life table method did not demonstrate a significant therapeutic effect of BCG.

However, our findings are compatible with those described in a recent paper by Owen *et al.* who also treated 6 dogs with BCG. Of the 6 BCG treated dogs 3 died 16, 25 and 52 weeks after amputation and 3 were still alive, 1 with metastasis 53 weeks after surgery and 2 with no evidence of disease 46 and 53 weeks after

surgery was performed. Survival times in 5 dogs receiving amputation alone ranged from 6–17 weeks (mean 11). A dose of 50–250 × 10^6 of viable organisms of BCG* was given by the intravenous route to all dogs irrespective of size at 1, 2, 4 and 8 weeks post-operatively.

The dose of BCG organisms administered was within the range of 10^6–10^7 viable organisms in both studies. Doses within the same range have been administered when treating human cancer patients with BCG [2, 3]. However, many studies in human patients have used considerably smaller doses of BCG. Some question still exists as to the possible effects of varying doses of viable BCG organisms on the rate of relapse and the disease-free period in human cancer patients [3]. Owen and Bostock did not determine if the survival times in the two groups of dogs were significantly different. Even though the survival times of 3 of the 5 control dogs used by Owen and Bostock were not reported the median test indicated that the BCG treated dogs had prolonged survival as compared to the controls ($\chi^2 = 7{\cdot}083$, $d.f. = 1$, $P < 0{\cdot}01$). When combining the test and control dogs from the present study with those of Owens there was a statistically significant difference in survival times ($\chi^2 = 6.783$, $d.f. = 1$, $P < 0{\cdot}01$). Although the strain of BCG and route of administration differed in the two studies the results were compatible.

Monitoring of *in vitro* CMR in tumor bearing dogs undergoing therapy with BCG and in controls gave information of clinical significance with respect as to how immunotherapy may influence the immune response. The findings in this study suggest that such an approach is possible, using the techniques employed. More trials are needed in which larger patient groups can be followed using standardized assay techniques in many independent laboratories.

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Plasma Dehydroepiandrosterone Sulfate, Androstenedione and Cortisol, and Urinary Free Cortisol Excretion in Breast Cancer*

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Abstract—*The concentrations of plasma dehydroepiandrosterone sulfate (DS), androstenedione (A2) and cortisol (C), and the urinary free cortisol (UFC) excretion were measured in* 24 *women with breast cancer before mastectomy and another* 45 *approximately* 1 *month after surgery, and in* 49 *cases of advanced breast cancer. The results were compared with those from a group of healthy women.*

Plasma DS was negatively correlated with age, and all 3 *breast cancer groups showed significantly reduced levels compared with age-comparable controls. Plasma A2 was also negatively correlated with age; no significant difference was found between the cancer patients and controls.*

The mean levels of plasma C and UFC in the breast cancer patients were normal, but 7 *of the* 49 (14%) *cases of advanced disease did have elevated UFC excretions.*

INTRODUCTION

IN A SERIES of publications, Bulbrook and his colleagues reported that subnormal excretions of urinary 11-deoxy-17-ketosteroids are associated with an increased risk of breast cancer recurrence after mastectomy [1] and the likelihood that the advanced disease is resistant to endocrine ablative therapy [2–4]. Further, they found that subnormal excretions of etiocholanolone and androsterone, the principal 11-deoxy-17-ketosteroids in urine, by apparently healthy women are indicative of an increased risk of breast cancer [5]. These observations suggest that it may be possible to use the determination of urinary androgens to identify those women in whom the breast cancer detection rate would justify annual screening by mammography, physical examination and, perhaps, thermography.

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There is a strong positive correlation between the urinary 11-deoxy-17-ketosteroid excretion and the concentration of dehydroepiandrosterone sulfate (DS) in plasma, but there have been conflicting reports regarding the plasma DS in breast cancer [6, 7]. The issue is of practical importance because if patients with a low excretion of androgen metabolites also have subnormal plasma DS, it follows that the assay of blood samples, instead of 24 hr urine collections, could be used when screening women for those with an increased breast cancer risk.

In the study reported here, the plasma DS has been determined in patients with early or advanced breast cancer, and healthy female controls. The opportunity was taken also to measure the plasma androstenedione (A2) and cortisol (C) levels, and the urinary free cortisol (UFC) excretion. Androstenedione is of special interest in the endocrinology of breast cancer because it is both an androgen and the principal precursor of estrogens in postmenopausal women [8]. The UFC provides a measure of the cortisol production rate [9]; it is elevated when there is an adrenal response to stress, which might, itself, alter the plasma DS and A2 levels.

MATERIAL AND METHODS

Twenty-four women with breast cancer, aged 44–69 years, were studied while in the hospital awaiting mastectomy, and another 45, aged 22–73 years, approximately 4–6 weeks after surgery. There were a further 25 patients with locally recurrent breast cancer, and 24 with distant metastases. Their ages ranged from 37–68 years. None of these cases had been treated by endocrine ablative surgery or additive hormone therapy, but some had received chemotherapy in the past.

Sixty-two healthy women aged 35–69 years constituted the control group for the plasma DS and A2 studies. None of these vo unt eers,who were attending the Milwaukee Breast Cancer Detection Clinic, had been receiving oral contraceptives, or any other form of steroids, for at least 6 months.

Plasma was obtained from heparinized blood taken between 8:00 and 10:00 a.m. A 24 hr urine collection for the determination of UFC was commenced as soon as the blood had been taken for the plasma steroid assays. The normal values for plasma C and UFC had been established in earlier studies; they were not influenced by age [10, 11]. Plasma DS [12] and A2 [13] were determined by radioimmunoassay, and plasma C and UFC by competitive protein-binding techniques [10].

Logarithmic transformation of the plasma steroid data were used because for a given age the steroid levels were closer to a logarithm-normal than a normal distribution. Natural logarithms were taken for this purpose. Statistical comparison between the various groups was by analysis of covariance.

RESULTS

The controls showed negative correlations with age for both plasma DS ($r = -0{\cdot}342$; $P < 0{\cdot}01$) and A2 ($r = -0{\cdot}497$; $P < 0{\cdot}001$), while the two steroids were positively correlated with each other ($r = 0{\cdot}544$; $P < 0{\cdot}001$). Because of the influence of age, appropriate regression lines were calculated for comparisons between the controls and the various breast cancer groups.

In Fig. 1 the regression lines of plasma DS on age are given. The preoperative and postoperative early breast cancer patients are shown separately, although there was no statistically significant difference between the two groups ($F = 0{\cdot}66$; $P > 0{\cdot}50$). The plasma DS concentrations for the patients awaiting mastectomy were lower than those of the controls ($F = 3{\cdot}82$; $P < 0{\cdot}05$), due to a significant difference in the two intercepts ($F = 7{\cdot}04$; $P < 0{\cdot}01$), but not in the slopes of the regression lines ($F = 0{\cdot}60$; $P > 0.25$). Similarly the plasma DS for the postoperative early breast cancer group was subnormal ($F = 3{\cdot}27$; $P < 0{\cdot}05$),

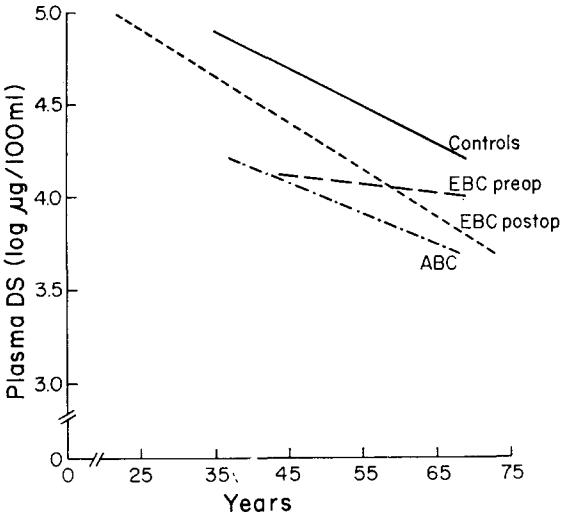

Fig. 1. Plasma DS in controls, early breast cancer before mastectomy (EBC preop), early breast cancer after surgery (EBC postop) and advanced breast cancer (ABC).
Regression lines of plasma DS on age were calculated; the statistical analysis is described in the text.

with intercepts differing from the controls ($F = 6{\cdot}38$; $P < 0{\cdot}05$). When the early breast cancer patients were combined into a single group, the differences from the control group were highly significant for the plasma DS ($F = 4{\cdot}79$; $P < 0{\cdot}01$) due to differences in the intercepts ($F = 9{\cdot}55$; $P < 0{\cdot}01$), but not the slopes ($F = 0{\cdot}02$; $P > 0{\cdot}50$).

The plasma DS concentrations of the patients with local recurrence did not differ from those with systemic metastases, and so they have been considered as a combined group in Fig. 1. This steroid was reduced markedly in the advanced breast cancer patients compared with the controls ($F = 10{\cdot}03$; $P < 0{\cdot}01$), and there was a highly significant difference in the intercepts ($F = 20{\cdot}01$; $P < 0{\cdot}001$). The two slopes of the regression lines were similar ($F = 0{\cdot}07$; $P > 0{\cdot}50$).

The results for the plasma A2 assays are summarized in Fig. 2. There was no difference between the two early breast cancer groups ($F = 0{\cdot}22$; $P > 0{\cdot}50$), nor between those with local recurrence or systemic metastases ($F = 0{\cdot}91$; $P > 0{\cdot}25$). Although it appeared that the early breast cancer patients had higher plasma A2 concentrations than the controls, the difference achieved only borderline statistical

significance ($F = 2{\cdot}97$; $P \simeq 0{\cdot}05$); the advanced breast cancer cases had unequivocally normal plasma A2 levels ($F = 0{\cdot}89$; $P > 0{\cdot}25$).

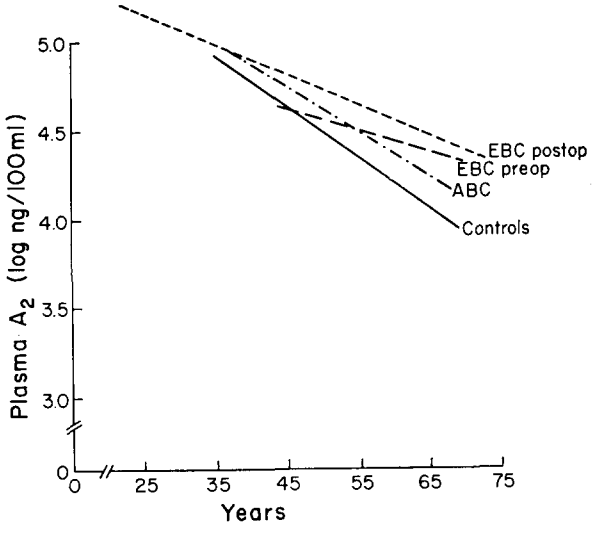

Fig. 2. Plasma A2 in controls, early breast cancer before mastectomy (EBC preop), early breast cancer after surgery (EBC postop) and advanced breast cancer (ABC).
Regression lines of plasma A2 on age were calculated; the statistical analysis is described in the text.

The plasma C and UFC results are summarized in Table 1. None of the breast cancer groups showed abnormal corticosteroid levels, although 7 of the 49 [14%] patients with advanced breast cancer did have an elevated UFC excretion.

Table 1. The plasma cortisol and urinary free cortisol in controls and breast cancer patients

Group (No.)	Plasma cortisol ln μg/100 ml	Urinary free cortisol μg/24 hr
Controls (31)	2·67 ± 0·36	57·3 ± 22.6
Early breast cancer: preop. (24)	2·72 ± 0·39	51·6 ± 29·5
Early breast cancer: postop. (45)	2·42 ± 0·66	47·7 ± 28·7
Advanced breast cancer (49)	2·53 ± 0·54	60·1 ± 31·9

No significant differences between groups for either the plasma cortisol or urinary free cortisol excretion.

DISCUSSION

Previous reports have indicated that the plasma DS in breast cancer may be influenced by the stage of the disease, and the time of blood sampling relative to mastectomy. Brownsey *et al.* [6] found the plasma DS to be reduced in advanced breast cancer, but normal in early breast cancer. In retrospect, it is unfortunate that they did not state whether blood was obtained from their early cases before or after mastectomy. The importance of this point emerged later when Wang *et al.* [7] measured the plasma DS in the same group of patients on the day before mastectomy, and again 10–14 days after surgery. Although the mean plasma DS for the preoperative samples was lower than that of controls, the difference was not statistically significant; in contrast, the mean postoperative plasma DS level was unequivocally subnormal.

In the present study, we did not find a difference between the DS levels in preoperative and postoperative plasma samples, and both groups of early breast cancer patients, like those with recurrent disease, had significantly reduced plasma DS concentrations compared with comparably aged controls. It should be noted that our study did differ from that performed by Wang *et al.* [7] in two respects: the two early breast cancer groups were not composed of the same patients, and our post-operative samples were obtained at least a month after mastectomy.

The mechanism by which mastectomy could affect the plasma DS is unclear. Wang *et al.* [7] postulated that preoperative psychological stress might be responsible, and noted that the urinary total 17-hydroxycorticosteroid excretion was significantly higher before mastectomy than afterwards. However, although the cortisol production rate is increased in advanced breast cancer, it is unchanged in patients awaiting mastectomy, and is not correlated with the urinary 17-hydroxycorticosteroids [14]. We chose, therefore, to measure UFC, which does give an indication of the cortisol production rate over a 24 hr period [9], and found it to be elevated only in some of the advanced breast cancer patients. The plasma cortisol was unchanged, which is in agreement with the results of Brownsey *et al.* [6], but at variance with the other reports that the plasma hydroxycorticosteroids are reduced in early breast cancer [15], and elevated in the advanced disease [16].

Recently, it was shown that plasma dehydroepiandrosterone is subnormal in both preoperative and postoperative early breast cancer [17], although the free steroid is considerably more sensitive to ACTH stimulation [18]. In conjunction with our results, these observations make it unlikely that psychological preoperative stress has any significant effect on plasma androgen levels.

Androstenedione is secreted from the ovaries

and adrenal glands; before the menopause the ovaries produce approximately 50% of the circulating steroid. In postmenopausal women adrenal A2 is the source of most of the plasma estrogens, and it has been postulated that increased aromatization of A2 to yield estrone is an important factor in the etiology of breast cancer [19]. However, in a study of 8 postmenopausal controls and 7 breast cancer patients, no difference was found in the conversion rate of A2 to estrone, although the cancer cases did show a small, statistically insignificant, elevation in metabolic clearance rate of A2 and a decrease in its plasma concentration [20].

We did not find reduced plasma A2 levels in breast cancer; indeed, the concentration of this steroid tended to be higher when compared with controls of similar age. Further investigations are underway in which we are measuring both plasma A2 and estrone in a group of postmenopausal breast cancer patients.

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Phase I–II Trial of Intramuscularly Administered Bleomycin

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Abstract—*Bleomycin was administered intramuscularly on one of four schedules in a phase I–II trial conducted by the Eastern Cooperative Oncology Group. A total of* 168 *evaluable patients received either bleomycin* 5 *mg/m² daily,* 5 *mg/m² weekly,* 20 *mg/m² weekly or* 40 *mg/m² weekly. The latter dose proved to be toxic and was discontinued. Comparable toxicity was seen with the other three schedules. The toxicity was qualitatively and quantitatively similar to that reported with other bleomycin doses, schedules and routes of administration except that no radiographic evidence of pulmonary toxicity was seen. Patients with hematologic malignancies and solid tumors responded equally well to the low dose and high dose bleomycin schedules. The highest response rates were seen in patients with testicular tumors and lymphomas. The median duration of response was* 1–2 *months.*

INTRODUCTION

BLEOMYCIN, an antitumor antibiotic produced from Streptomyces verticillus, has therapeutic activity in a variety of human neoplasms [1–3]. Despite intensive investigation, however, the optimal dose and schedule of bleomycin administration for maximal therapeutic benefit with minimal toxicity is not known. The most commonly used dose and schedule has been 15 mg/m² twice weekly intravenously (range of 4 mg/m² to 35 mg/m² [1, 4]. Other evaluated schedules include weekly [5], daily to toxicity [2], daily intermittent [3, 6] and continuous intravenous infusions in cyclic courses [7]. A range of drug doses have been used with these schedules.

In 1971 the Eastern Cooperative Oncology Group initiated a phase I–II bleomycin study. The aims of this study were to evaluate a less commonly used route of bleomycin administration (intramuscular) to compare toxicity and therapeutic benefit of low dose bleomycin (5 mg/m²) on a daily and weekly schedule, and to establish an optimal bleomycin dose for weekly intramuscular administration.

MATERIAL AND METHODS

A total of 168 patients having a variety of histologically proven inoperable malignancies were entered on study. Ten patients were subsequently declared invalid either because of

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Other participating institutions include: Albany Medical College, Albany, N.Y. (CA 06594); University of Alberta, Edmonton, Alberta, Canada (CA 13314); Boston University Medical Center, Boston, Mass. (CA 11109); Mt. Sinai Hospital Medical Center, Chicago, Ill. (CA 14144); Georgetown University School of Medicine, Washington, D.C. (CA 02824); Hahnemann Medical College, Philadelphia, Pa. (CA 13611); Jefferson Medical College, Philadelphia, Pa. (CA 14215); Lahey Clinic Foundation, Boston, Mass. (CA 12880); Maimonides Medical Center, Brooklyn, N.Y. (CA 05588); Centre Hospitalier Universitaire Lariboisier, Paris, France; Roswell Park Memorial Institute, Buffalo, N.Y. (CA 12296); and Tufts University School of Medicine, Boston, Mass. (CA 07190).

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protocol violations or early death unrelated to therapy leaving 158 evaluable patients. All patients had measurable or evaluable disease. Seventy-six (48%) of the 158 evaluable patients had had previous chemotherapy. No patient had received radiation therapy during the month prior to study and disease progression of the irradiated lesion was required for protocol entry if that was the only area of measurable or evaluable disease. All patients had adequate hematologic, renal and hepatic function, and none had significant underlying pulmonary disease. All patients volunteered informed consent.

Laboratory and clinical studies

The following tests were to be performed weekly: determination of WBC, differential, platelet, and reticulocyte counts and timed inspiratory and expiratory vital capacity. Tests done every 2 weeks included BUN and/or creatinine, alkaline phosphatase, chest X-ray, tidal volumes and blood gases. Tumor measurements using the product of the longest perpendicular tumor diameters were done biweekly.

Study plan

Four treatment schedules were evaluated.

A. Daily 5 mg/m^2 i.m. for 5–7 days weekly
B. Weekly 5 mg/m^2 i.m.
C. Weekly 20 mg/m^2 i.m.
D. Weekly 40 mg/m^2 i.m.

The total dose of bleomycin administered was not to exceed 200 mg/m^2. For patients treated on schedules B and C, if no response was seen after one month their dose could be increased to 40 mg/m^2. When a 50% or greater response was achieved bleomycin was stopped for one month and was then reinstituted at the original dose and schedule.

Standard response criteria were used with partial response indicating a 50% or greater shrinkage in the product of the longest perpendicular tumor diameters without progression of any lesion or the appearance of a new lesion. Progression indicated a 50% or greater increase in the product of tumor diameters or the appearance of new lesions.

RESULTS

Table 1 indicates the drug toxicity seen in regimens A, B and C. Regimen D (bleomycin 40 mg/m^2 i.m. weekly) was discontinued early in the study after two patients expired in the first week of therapy after developing severe and prolonged chills and fever with the initial dose. Chills and fever were more often observed at higher bleomycin doses occurring in 20% of patients on schedule C as opposed to 7 and 9% of patients respectively on schedules A and B. These symptoms generally appeared within several hours of bleomycin administration and were relieved, in large part, by antipyretics. There did not appear to be a predisposition for patients with hematologic malignancies to develop any greater toxicity than did solid tumor patients.

Skin and mucous membrane toxicity occurred with the same frequency on all three schedules. Mild to moderate skin toxicity included erythematous, painful, indurated areas on the hands, elbows and other pressure areas of the skin. More severe skin toxicity consisted of a diffuse morbilliform skin eruption. Mucous membrane toxicity in the form of stomatitis was neither severe nor dose limiting in any patient. Alopecia was infrequent and was never complete.

Nausea and vomiting following bleomycin was severe in only two of the 158 patients in this study. Both patients were treated on the daily bleomycin schedule and this toxicity was dose limiting in both patients. Hematologic toxicity with white blood cell counts and/or platelet counts transiently below 1500/mm^3 and 50,000/mm^3 respectively were noted in one patient each on schedules B and C. Over 90% of patients on all schedules had no evident hematologic toxicity.

Respiratory toxicity was clinically manifested by development of increased cough and shortness of breath with or without the presence of bibasilar rales on physical exam. These symptoms were classified as mild to moderate in 5 patients treated on schedule A, and 4 patients on schedule C, and were severe in 2 patients on schedule A and in one patient on each of the other two schedules. No patients developed radiographic evidence of pulmonary infiltrates or fibrosis. Pulmonary function studies were only performed sporadically on these patients but where information was available, no correlation between pulmonary function abnormalities and clinical symptoms and/or signs was noted.

Tumor response

Tumor responses were noted in each of the four treatment schedules (Table 2). The response by tumor site is indicated in Table 3. Responses in lymphoma patients occurred on all of the schedules. Four of the five testicular cancer patients who responded were treated

Table 1. *Bleomycin toxicity*

Toxicity parameter	Schedule A 5 mg/m² daily (45 patients) None	Mild to moderate	Severe	Schedule B 5 mg/m² weekly (32 patients) None	Mild to moderate	Severe	Schedule C 20 mg/m² weekly (71 patients) None	Mild to moderate	Severe
Chills and fever	93*	7	0	91	6	3	80	6	14
Skin and mucous membrane	80	11	9	78	13	9	85	10	5
Vomiting and diarrhea	82	13	5	97	3	0	86	14	0
Hematologic	100	0	0	94	3	3	96	2	2
Respiratory	84	12	4	97	0	3	93	6	1

*% of treated patients with the indicated degree of toxicity.

Table 2. *Tumor response to bleomycin*

Schedule	Response Complete	Partial	No change	Progression	Total
A	0 (0%)	9 (20%)	21 (47%)	15 (33%)	45
B	1 (3%)	4 (13%)	10 (31%)	17 (53%)	32
C	0 (0%)	8 (11%)	27 (38%)	36 (51%)	71
D	0 (0%)	2 (20%)	4 (40%)	4 (40%)	10
Total	1 (1%)	23 (15%)	62 (39%)	72 (46%)	158

Schedule A—5 mg/m² daily, B—5 mg/m² weekly, C—20 mg/m² weekly, D—40 mg/m² weekly. All drug dosages were administered i.m.

Table 3. *Bleomycin response by tumor type*

Tumor type	Response Complete	Partial	No change	Progression	Total
Lymphoma	1	5	8	4	18
Lung		1	3	6	10
Breast		1	3	1	5
Cervix		2	3	4	9
Testicular		5	1		6
G.I.		2	10	12	24
Head and Neck		7	24	22	53
Ovary				6	6
Other			10	17	27
Total	1	23	62	72	158

on the daily 5 mg/m^2 schedule. The time from the start of therapy until tumor response in the 24 responding patients is noted in Table 4. Sixteen (67%) patients who responded achieved their reponse in less than 1 month. The duration of response is shown in Table 5. Nine of 24 responses lasted over two months.

Table 4. *Time to response*

Number of days to start of response	Number of patients*
1–14	6
15–28	10
29–42	6
43 and over	2

*Total = 24

Table 5. *Duration of tumor response*

Time	Number of patients*
< 1 month	8
1–2 months	6
2–3 months	5
3 months and over	4
Unknown	1

*Total = 24.

Dose escalation

Following the rules of the protocol, 27 patients on schedule C (20 mg/m^2 weekly) and 6 patients on schedule B (5 mg/m^2 weekly) had their dose increased to 40 mg/m^2 after 1 month of treatment. For all 33 patients the dose increase was stopped before the completion of a further month's treatment. One patient on schedule C achieved a partial tumor response after the dose increase. Several patients had further toxicity following the dose increase but in only 2 cases was this severe (one patient had chills and fever, the other had mental confusion).

DISCUSSION

The toxicity seen with intramuscular administration of bleomycin given as 5 mg/m^2 daily or weekly or as 20 mg/m^2 weekly was both qualitatively similar to that reported with a variety of other bleomycin schedules [1–6] and with intravenous or intraarterial drug administration [1]. Administration of 40 mg/m^2 of bleomycin weekly was clearly too toxic for continued use. It was interesting that the amount of drug toxicity on the above three schedules was similar with patients on the low dose schedule (5 mg/m^2 weekly) having no fewer toxic reactions than patients on 5 mg/m^2 daily or 20 mg/m^2 weekly. The median bleomycin dose received was 125 mg/m^2, 40 mg/m^2 and 160 mg/m^2 on the daily, low dose weekly and high dose weekly schedules respectively. At these dose levels one would expect radiologic manifestations of pulmonary toxicity in 4% of treated individuals [1]. No definite radiographic bleomycin pulmonary toxicity was noted in this study. Further study of large numbers of patients treated with intramuscular bleomycin will be necessary to determine whether the risk of pulmonary toxicity is decreased by this route of drug administration.

The therapeutic responses were primarily in tumor types known to be responsive to bleomycin therapy, i.e., lymphomas, testicular tumors, and epidermoid carcinomas of the lung, head and neck, breast and uterine cervix. Responses were seen with all bleomycin schedules used. A therapeutic superiority for any of the schedules cannot be demonstrated as patients with responsive tumors were not uniformly distributed. Also, patient performance status, another important prognostic variable for response [8], was unbalanced with 58% of patients receiving 20 mg/m^2 of bleomycin weekly being ambulatory at the start of treatment as opposed to only 40% ambulatory patients on the other two schedules. Response rates for bleomycin given intramuscularly appear comparable to those obtained with bleomycin given intravenously. Further, it appears that solid tumors as well as hematologic malignancies may respond equally to low dose bleomycin administration and to high drug doses. Response durations in this study were relatively short (median duration 1–2 months) suggesting that bleomycin usefulness will be in combination chemotherapy regimens.

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The Inhibitory Effects of a 4-Hydroxy-Pentenal: Cysteine Adduct Against Sarcoma 180 Cells in Mice*

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Abstract—*The carcinostatic activity of a 4-hydroxy 2,3-trans-pent-en-1-al(HPE): cysteine adduct was studied against the ascitic form of Sarcoma 180 both* in vitro *and* in vivo. *The effect of HPE treatment* in vitro *(at concentrations in excess of 0·16 mM) was to depress primarily the incorporation of ^{3}H-thymidine into DNA; ^{3}H-uridine and ^{3}H-leucine incorporations into RNA and protein respectively were considerably less affected. The addition of 0·16 mM cysteine before treatment of Sarcoma 180 cell suspensions with HPE provided partial protection in relation to the depression of ^{3}H-thymidine incorporation. Tumour cell suspensions were preincubated* in vitro *for 30–120 min at 37°C with 0·04–1·28 mM HPE, HPE-cysteine or cysteine before the addition of ^{3}H-thymidine. The effectiveness of different types of preincubation procedure, in depressing the subsequent incorporation of ^{3}H-thymidine into Sarcoma 180 tumour cells* in vitro, *were compared. The results obtained indicate that HPE-cysteine was 4–7 times less effective on a molar basis than treatment of tumour cells* in vitro *with HPE alone.*

The LD_{50} *in mice for a single i.p. injection of HPE-cysteine was 362 mg/kg body weight (expressed as mg HPE); thus the adduct is approx. 7 times less toxic than the free aldehyde injected i.p. Extensions in survival time were produced by i.p. injection of increasing doses of HPE-cysteine* in vivo, *in mice bearing the ascitic form of Sarcoma 180. Treated animals received, on days 3–7 following transplantation, a single daily dose of the HPE-cysteine adduct equivalent to a dose of HPE alone of 8–256 mg/kg body weight. Significant increases in survival time were obtained at doses of HPE-cysteine in excess of 32 mg/kg body wt./day. Mice treated with the adduct responded with increases in survival time, compared to untreated control animals, of 50%, 94% and 129% at doses of the adduct equivalent to doses of HPE alone of 64, 128 and 256 mg/kg body wt./day respectively.*

The results obtained are consistent with the view that the extended breakdown of an adduct of HPE and cysteine, releasing the reactive aldehyde, increases the half-life of HPE both in vitro *and* in vivo.

1. INTRODUCTION

In a previous publication [1] we reported the results obtained by treating murine tumour cells with added HPE *in vitro* and the increases in survival time observed on re-injection of the treated cells into groups of mice. The data demonstrated that HPE was a relatively potent carcinostatic agent *in vitro* against the tumour lines tested. Preliminary work [2] *in vitro* has indicated that HPE (0·16–0·20 mM) severely depressed ^{3}H-thymidine incorporation into DNA in Ehrlich carcinoma, Gardner lymphosarcoma, and Sarcoma 180; ^{3}H-uridine and ^{3}H-leucine incorporation into RNA and protein were considerably less affected. The addition of cysteine before treatment of the above tumour lines with added HPE provided partial protection [3].

Treatment *in vivo* of these cell lines with multiple i.p. injections of HPE resulted in no significant increase in survival time, compared to untreated controls, for Ehrlich carcinoma

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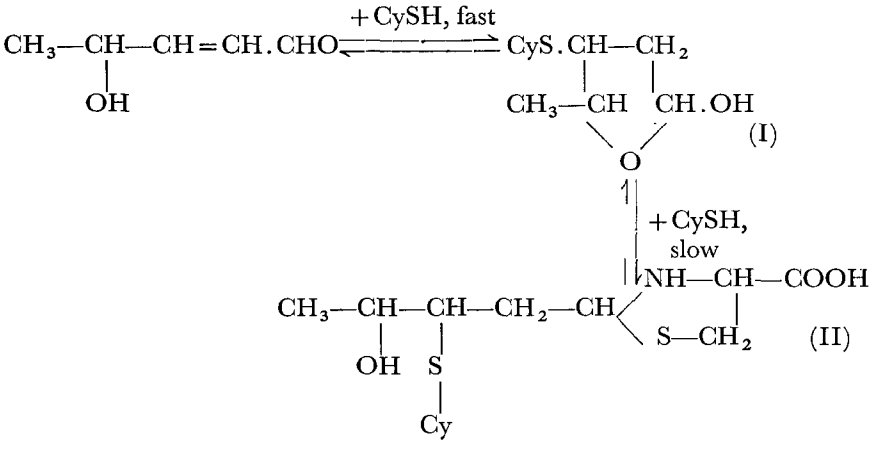
and Gardner lymphosarcoma; Sarcoma 180 responded with a moderate inhibition of tumour growth [1]. The relatively short half-life ($t_{\frac{1}{2}}$) of HPE is probably of major significance to the poor carcinostatic activity of the compound when administered other than peritumourally [4–7]. One possible way of increasing the $t_{\frac{1}{2}}$ of HPE *in vivo* may be via the formation of an adduct of the aldehyde with cysteine. Guidotti *et al.* [8] suggested that aliphatic aldehydes condense with exogenous cysteine forming thiazolidine-4-carboxylic acid derivitives [8–10], thus depriving cells of that amino acid. These effects received confirmation in cultured mammalian cells [11] and in bacteria [12]. The resulting inhibition of protein synthesis could then explain the encouraging results reported by a number of other workers [13–15] on the survival of leukaemia and Ehrlich ascites tumour-injected rodents upon treatment with aliphatic aldehydes.

Esterbauer *et al.* [16] have recently demonstrated that HPE forms a thiazolidine-4-carboxylic acid derivative with cysteine in the following manner:



The rate constants for the reaction of HPE with cysteine increases with increasing pH, approaching a maximum at pH10. The rate limiting step at any pH is the addition of the sulphydryl anions $^{-}SCH_2CH(NH_3^{+})COO^{-}$ and $^{-}SCH_2CH(NH_2)COO^{-}$ to the C=C bond of HPE. The thiazolidine derivative (II) loses a cysteine residue fairly rapidly and the resultant Michael-type monoadduct then dissociates further in a slower reaction to release the aldehyde and cysteine. A purified HPE-cysteine adduct (molar ratio 1:1) of defined structure has been shown to possess significant carcinostatic activity against the ascitic form of Ehrlich carcinoma in mice [17]. Under the reaction conditions employed herein (see materials and methods) the present authors cannot be certain of the precise structure or homogeneity of the HPE cysteine adduct investigated in this study; it is important, however, to note that the adduct tested here was found to have potent biological reactivity. We have investigated the effects of such an adduct of HPE and cysteine in two ways:

(1) on the incorporation *in vitro* of ^{3}H-thymidine into DNA of suspensions of Sarcoma 180 tumour cells;
(2) the extension in survival time produced by i.p. injection of increasing doses of the adduct *in vivo*, in mice bearing the ascitic form of Sarcoma 180.

2. MATERIAL AND METHODS

L-Cysteine hydrochloride, sodium chloride, tris(hydroxymethyl) methylamine, perchloric acid, concentrated hydrochloric acid and Whatman GF/C glass fibre discs were purchased from B.D.H. Chemicals Ltd., Poole, England. All reagents were of Analar or Aristar grade. Scintillation grade PPO (2,5-diphenyloxazole) and POPOP (1,4-Di[2-5-phenyloxazoyl] benzene) were purchased from Koch-Light Laboratories Ltd., Colnbrook, England. Tissue culture medium (TC 199) and horse serum 5 [inactivated] (HS5) were obtained from Wellcome Reagents Ltd., Beckenham, England. Radioactive precursors listed below came from the Radiochemical Centre, Amersham, England. Female CBA/CA mice, produced from an inbred colony maintained at Brunel, were used throughout.

Tumour maintenance

Sarcoma 180 in the ascitic form was maintained in female CBA/CA mice (20–25g). Routine passaging and harvesting of tumour cells occurred on the 7th day following intraperitoneal injection of 2×10^{6} cells/animal.

Methods

Preparation of 4-hydroxy-2,3-trans-pent-en-1-al (HPE). The compound was synthesized and purified as previously described [1].

Preparation of HPE-cysteine adduct. A solution of HPE (100 mg, 1×10^{-3} mole) in chloroform (18 ml) was shaken with a solution of L-cysteine hydrochloride (473 mg, 2×10^{-3} mole) in water (0·5 ml) until HPE could no longer be detected in the chloroform layer by u.v. spectroscopy (approx. 1 hr.). The mixture was left to stand overnight after which the chloroform was removed on a rotary evaporator. Thin layer chromotography (TLC) on Silica gel with the solvent system *n*-butanol: acetic acid: water (12:3:5) showed one major product clearly separated from the excess cysteine. Preparative TLC (1 mm layer thickness) of the product mixture using the same solvent system produced a white solid (50 mg), insoluble in all common pure solvents tested except water.

Preparation of HPE-cysteine adduct for testing in vitro. (i) The HPE-cysteine adduct (41·6 mg, $1{\cdot}28 \times 10^{-4}$ mole) purified by TLC was dissolved in 0·9% saline (1 ml) and serially diluted to give a range of concentrations (1·3–41·6 mg/ml, 4-128 mM). The addition of 0·1 ml of the above HPE-cysteine solutions to tumour cell suspensions (10 ml) gave final concentrations of 0·04–1·28 mM.

(ii) A graded series of HPE solutions (0·4–12·8 mg, $0{\cdot}04$–$1{\cdot}28 \times 10^{-5}$ mole) in 0·9% saline (0·5 ml) were mixed with a similar graded series of solutions of L-cysteine hydrochloride (1·26–40·5 mg, $0{\cdot}08$–$2{\cdot}56 \times 10^{-5}$ mole) in 0·9% saline (0·4 ml) and incubated with shaking for 1 hr at 37°C. The resulting mixtures were neutralized to pH 7·2 and made up to 1·0 ml. As in (i) above, the final concentrations of the HPE-cysteine adduct in tumour cell suspensions (10 ml) were 0·04–1·28 mM.

Radioactive precursor incorporation procedures. The following quantities of labelled precursors were used per ml of tumour cell suspension: thymidine-6-H, 5·0 Ci/mmole, 0·25 μCi/ml; uridine-5-^{3}H, 5·0 Ci/mmole, 0·25 Ci/ml; DL-leucine-4,5-^{3}H, 1·0 Ci/mmole, 2·5 μCi/ml.

Ascites tumour cells were harvested from the peritoneal cavity of the mouse into 5 ml of sterile 0·9% saline containing 0·1 ml heparin. The cells were then washed with sterile "lysing buffer" (7·47 g NH_4Cl; 2·06 g Tris–HCl in 1 l. of distilled water brought to pH 7·2 with 11·6 M HCl) to remove erythrocytes and sedimented at 700 g_{av} for 5 min. The process was repeated until an erythrocyte-free pellet was produced. The pellet was gently resuspended in 5 ml sterile 0·9% saline and a count taken on a Coulter Model D Particle Counter. The cells were then resuspended in tissue culture medium 199: horse serum 5-(60:40) at a concentration of 1×10^6 cells/ml. Aliquots (10 ml) of the tumour cell suspension were removed into sterile, stoppered Ehrlenmeyer flasks (25 ml) and were incubated with shaking at 37°C before the addition of HPE, HPE-cysteine, or solvent as control. Cells were then further incubated with shaking for 30–120 min in the presence of these drugs, before the addition of labelled precursors. At time intervals 1 ml samples were removed and filtered onto Whatman GF/C glass fibre discs wetted with 0·9% saline, 10 volumes of 0·2 N perchloric acid, and a further 10 volumes of 0·9% saline. The filters were placed in scintillation vials and dried at 100°C for 1 hr. 5 ml of scintillation fluid (Toluene: 4g/l PPO [2,5-phenyloxazole]; 0·2 g/l POPOP [1,4-Di(2-5-phenyloxazoyl)benzene]) was added directly to the dried filters and the samples counted in a Packard Tri-carb Model 2425 liquid scintillation spectrometer.

Preparation of HPE-cysteine adduct for testing in vivo. A solution of HPE (410 mg, $4{\cdot}1 \times 10^{-3}$ mole) in 0·9% saline was mixed with a solution of L-cysteine hydrochloride (1290 mg, $8{\cdot}2 \times 10^{-3}$ mole) in 0·9% saline (7 ml) and incubated with shaking for 1 hr at 37°C. This mixture was neutralized to pH 7·2 and made up to 16 ml. Assuming that the HPE-cysteine adduct breaks down completely over an extended period, this would produce 25·6 mg/ml of free HPE. Serial dilution in 0·9% saline produced a range of concentrations of the adduct (equivalent to a solution of HPE alone of 0·8–25·6 mg/ml). Treated animals received a solution of HPE-cysteine adduct in 0·9% saline on the basis of 10 ml of drug solution per kg body weight.

In vivo *testing of the HPE-cysteine adduct.* The method employed has been described previously [1].

3. RESULTS

Effects on the incorporation of ^{3}H-precursors into Sarcoma 180 cell suspensions treated with added HPE in vitro

Sarcoma 180 ascites cells were incubated at 37°C in tissue culture medium 199: horse serum in the presence and absence of HPE for 30 min and ^{3}H-precursors added to detect drug-induced alterations in bio-synthetic reactions. The incorporation of ^{3}H-thymidine (TdR) appears to be primarily affected at doses

of HPE in excess of 0·16 mM. Total incorporation of labelled thymidine into DNA is reduced to approximately 10% of the control level at 0·16 mM HPE, whereas ^{3}H-uridine (Ud) incorporation into RNA and ^{3}H-leucine (Leu) incorporation into protein are reduced to approximately 50% of the control incorporation at 0·16 mM HPE. The depression of incorporation of these labelled precursors into Sarcoma 180 tumour cells, produced by exposure to the drug, is concentration dependent.

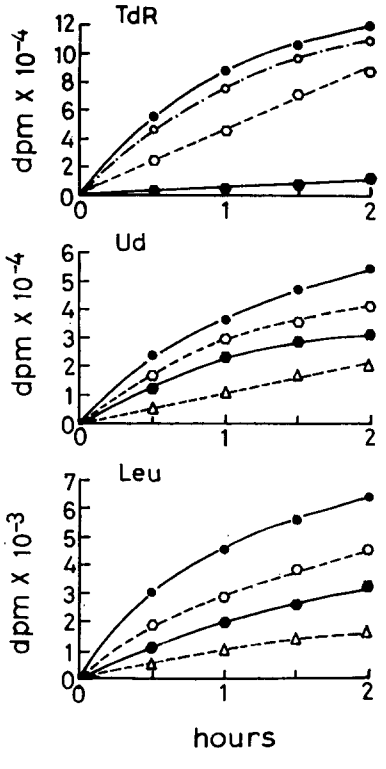


Fig. 1. Effect of 30 min preincubation of Sarcoma 180 cells (1 x 10⁶/ml) with 0·04–0·32 mH HPE per ml of cell suspension on the subsequent incorporation of isotopic precursors for cell components over 2 hr at 37°C. ^{3}H-thymidine (TdR) was incorporated into DNA; ^{3}H-uridine (Ud) principally into RNA; ^{3}H-leucine (Leu) into protein. ●—●, control cultures; ○—·—○, 0·04 mH HPE; ◇– – –◇, 0·08 mM HPE; ◆ — ◆ 0·16 mH HPE; △– – –△, 0·32 mM HPE. All points represent the mean of at least 3 expts.

Figure 1 illustrates that for each doubling of the concentration of added HPE the incorporation of ^{3}H-uridine and ^{3}H-leucine declines by approx. 50%. However, the uptake of ^{3}H-thymidine into DNA of the tumour cells exhibits a sigmoidal response to HPE treatment. Since the effect of HPE treatment is to primarily depress incorporation of labelled thymidine into DNA, this tritiated precursor was selected to investigate the protective effects of cysteine against added aldehyde and the response of tumour cells *in vitro* to pretreatment in various ways with the adduct formed between HPE and cysteine.

Comparison of the effects of HPE and HPE-cysteine on the incorporation of ^{3}H-thymidine into Sarcoma 180 *tumour cells* in vitro

The types of preincubation at 37°C of tumour cells with different drug regimes before the addition of ^{3}H-thymidine (TdR) are shown in Fig. 2. The effectiveness of different types of preincubation were compared to two control procedures: (1) and (2).

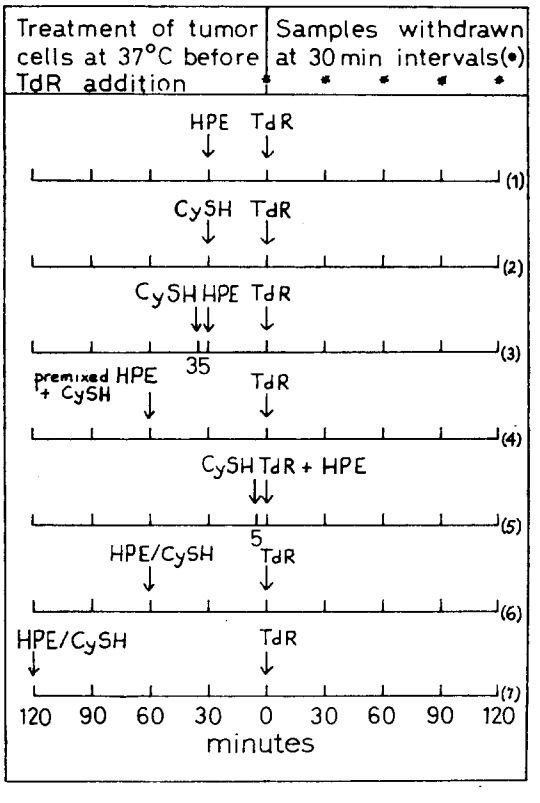


Fig. 2. Plan of various types of preincubation of Sarcoma 180 cells (1 x 10⁶/ml) at 37°C before addition of ^{3}H-thymidine (TdR). Samples were withdrawn at 30 min intervals over 2 hr for radioactivity assay. The types of procedure were (1) *0·04–1·28 mM HPE per ml of cell suspension added 30 min before TdR addition;* (2) *0·04–1·28 mM cysteine per ml of cell suspension added 30 min before TdR addition;* (3) *0·16 mM cysteine added at 35 min followed by 0·04–1·28 mM HPE added 30 min before TdR addition;* (4) *0·04–1·28 mM HPE premixed with 0·08–2·56 mM cysteine added 60 min before TdR addition;* (5) *0·16 mM cysteine added at 5 min and 0·04–1·28 mM HPE and TdR added simultaneously;* (6) *0·04–1·28 mM HPE-cysteine added 60 min before TdR addition;* (7) *0·04–1·28 mM HPE-cysteine added 120 min before TdR addition.*

The dose–response relationship of these two treatments is shown in Fig. 3 and repeated for comparative purposes in each dose–response plot. Treatment of tumour cells with HPE

alone or cysteine, as above, depresses ^{3}H-thymidine incorporation compared to untreated controls and produces a sigmoidal response.

In preincubation procedure (3) the dose–response plot is shifted slightly to the right, indicating a small degree of protection by the cysteine against added aldehyde. The addition of 0·16 mM cysteine alone to the cell suspension only reduces the rate of incorporation of precursor by 4% compared to solvent treated controls.

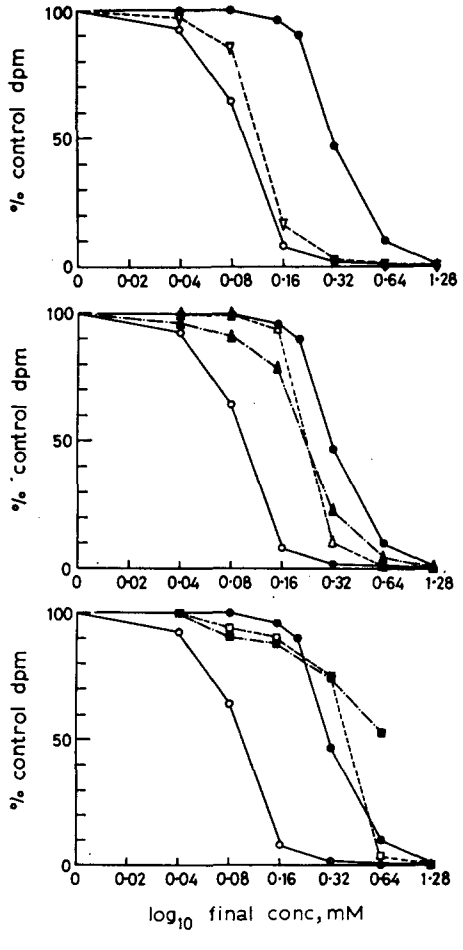


Fig. 3. Effect of drug concentration during preincubation at 37°C on the subsequent rate of incorporation of ^{3}H-thymidine into DNA of Sarcoma 180 tumour cells (1×10^6/ml). Procedures as outlined in Fig. 2 (1) ○—○, HPE added alone 30 min before TdR addition; (2) ●—●, cysteine alone added 30 min before TdR addition; (3) ▽– – –▽, 0·16 mM cysteine added at 35 min followed by HPE at 30 min before TdR addition; (4) △– – –△, HPE premixed with cysteine and added 60 min before TdR addition; (5) ▲—·—▲, 0·16 mM cysteine added at 5 min before the simultaneous addition of HPE and TdR; (6) ■—·—■, HPE-cysteine added 60 min before TdR addition; (7) □– –□, HPE-cysteine added 120 min before addition of TdR. Plotted points represent the mean values obtained from at least 3 experiments.

Premixing the HPE and cysteine at a molar ratio of 1:2 (see Materials and Methods) as in preincubation procedure (4) produced a much more pronounced shift of the dose–response plot towards that of cells treated with cysteine alone. A broadly similar effect was obtained in preincubation procedure (5). It is significant that the responses of tumour cells to preincubation procedures (4) and (5) lie between the plot indicated as controls, procedures (1) and (2). The result of procedure (5), when compared to that obtained in procedure (3), suggests that the preincubation period of 30 min is sufficient to allow a proportion of the HPE-cysteine adduct formed *in situ* to decay, releasing HPE and cysteine.

This suggestion receives confirmation when comparing the response of tumour cells to the addition of the HPE-cysteine adduct (purified by TLC as in Materials and Methods). Preincubation procedure (6) illustrates the response obtained from a 60 min incubation period with the purified adduct. Comparing the responses produced on a dose for dose basis, the adduct is less effective than treatment of tumour cells with cysteine alone at concentrations in excess of 0·20 mM. Increasing the preincubation period when Sarcoma 180 cells are exposed to the purified HPE–cysteine adduct to 120 min produces a similar response up to 0·32 mM. Thereafter it declines rapidly to 3% of the untreated control at 0·64 mM. The adduct appears to be breaking down with time in the medium, releasing two moles of cysteine for every mole of HPE. It is possible that cysteine and HPE, at concentrations in excess of 0·32 mM, act synergistically in depressing ^{3}H-thymidine incorporation into DNA.

These results are summarized in Table 1. A quantitative comparison of different preincubation treatments can be made if we compare the dose required (mM) to reduce ^{3}H-thymidine incorporation to 50% or 10% of the untreated control incorporation (ID_{50} and ID_{90}). The ratio of the ID_{50} or ID_{90} for tumour cells treated with HPE alone to that for cells treated in any other manner is an index of molar effectiveness of that treatment. The effects of procedure (3) are comparatively slight. Preincubation procedures (4) and (5) are broadly equivalent. To obtain the same degree of depression of ^{3}H-thymidine incorporation compared to treatment of HPE alone procedure (1) between 2 to 3 times the dose is required. Similarly procedures (6) and (7) are between 4 and 7 times less effective on a molar basis than treatment of Sarcoma 180 cells with HPE alone.

Activity of the HPE-cystine adduct against Sarcoma 180 in vivo

The LD_{50} in mice for a single i.p. injection of HPE, as the free aldehyde, is 55 mg/kg body wt. [1]. HPE injected i.p. in 4 equal doses spaced at 2-hourly intervals, gives an LD_{50} in CBA/CA mice of 180 mg/kg body wt./day [1]. Injection i.p. of the HPE-cysteine adduct into mice produced an LD_{50} value of 362 mg/kg body wt./day (expressed as mg HPE assumed to be released from the adduct). Thus the HPE-cysteine adduct is much less toxic than the free aldehyde injected i.p.; in comparison to a single i.p. injection of HPE as the free aldehyde, HPE-cysteine is approx. 7 times less toxic.

The extensions in survival time produced by i.p. injection of increasing doses of the HPE-cysteine adduct *in vivo*, in mice bearing the ascitic form of Sarcoma 180, are given in Table 2. Significant increases in survival time,

Table 1. *Quantitative comparison of different preincubation procedures on the subsequent incorporation of* ^{3}*H-thymidine (TdR) into DNA of Sarcoma* 180 *tumour cells* (1×10^6/*ml*) *at* 37°*C. Preincubation treatments* 2–7 *are compared to treatment* 1 (*HPE added at* 30 *min before TdR addition*). *The* ID_{50}(*mM*) *and* ID_{90}(*mM*) *values are derived from the dose–response plots in Fig.* 3 *and are doses required to depress* ^{3}*H-thymidine incorporation to* 50% *and* 10% *of solvent treated controls*

Procedure number (see Fig. 2)	Procedural summary: preincubation of tumour cells at 37°C before TdR addition	Total incorporation of controls 120 min after TdR addition, dpm × 10^{-4}/ml ± SEM	ID_{50} (mM)	ID_{90} (mM)	$\frac{ID_{50}}{ID_{50}*}$	$\frac{ID_{90}}{ID_{90}\dagger}$
1.	HPE added, 30 min preincubation	12·22 ± 0·18 (6)	0·096*	0·157†	1·00	1·00
2.	Cysteine added, 30 min preincubation	10·09 ± 0·07 (3)	0·311	0·640	3·23	4·08
3.	Cysteine added at 35 min, followed by HPE added at 30 min before TdR	11·93 ± 0·37 (3)	0·114	0·215	1·19	1·37
4.	Premixed cysteine and HPE added at 60 min before TdR	10·76 ± 0·59 (3)	0·230	0·320	2·40	2·04
5.	Cysteine added at 5 min, followed by simultaneous addition of HPE + TdR	8·96 ± 0·27 (3)	0·230	0·520	2·40	3·31
6.	HPE-cysteine adduct added at 60 min before TdR addition	9·35 ± 0·09 (3)	0·640		6·67	
7.	HPE–cysteine adduct added at 120 min before TdR addition	9·74 ± 0·37 (3)	0·410	0·610	4·27	3·38

Table 2. In vivo *carcinostatic data for Sarcoma* 180 *in female CBA/CA mice* (20–25 *g*). 1×10^6 *cells injected i.p. per mouse. Treated animals received, on days* 3–7 *following transplantation, a single daily dose of the HPE-cysteine adduct equivalent to dose of HPE alone of* 8–256 *mg/kg body weight*

Daily dose of HPE-cysteine in mg/kg body wt.	Day of death	Range of death	Average day of death ± SEM	% Increase in survival time	*p*-value (tailed *t* test)
Untreated controls	16 16 16 17 17 17 17 17 17 17 17 17 17 17 17 17 18 18 18 18	16–18	17·05 ± 0·14		
8	16 17 17 17 17	16–17	16·80 ± 0·20	−1·5	$P \geqq 0.40$
16	16 16 17 17 17	16–17	16·60 ± 0·25	−2·6	$P \geqq 0.10$
32	17 19 19 19 19*	17–19	18·60 ± 0·40	+9·1	$P < 0.001$
64	22*23*25*25*26*26*26*26*28*28*	22–28	25·50 ± 0·60	+49·6	$P < 0.001$
128	28*29†30†32†32†33†33†38†38†38†	28–38	33·10 ± 1·19	+94·1	$P < 0.001$
256	32†36†37†37†38†38†38†42†46†46†	32–46	39·00 ± 1·40	+128·7	$P < 0.001$

*Both ascitic fluid and solid tumour present on death.
†Only solid tumour present on death.

compared to untreated controls, were obtained at doses of HPE-cysteine in excess of 32 mg/kg body wt./day ($P < 0{\cdot}001$). Mice treated with HPE-cysteine responded with increases in survival time of 50%, 94% and 129% at doses of the adduct equivalent to doses of HPE alone of 64, 128 and 256 mg/kg body wt./day respectively. Animals receiving 64 mg/kg body wt./day of the adduct all had both ascitic fluid and a solid tumour present on death. Only one mouse in the group of 10 animals treated 128 mg/kg body wt./day with HPE-cysteine died with both a solid tumour and ascitic fluid present. The remaining animals in this group, together with mice receiving 256 mg/kg body wt./day of the adduct, all died with a proliferated solid tumour and the absence of ascites in the peritoneal cavity. Solid tumours were detected initially as small subcutaneous nodules (2–3 mm. dia) at or near the tumour injection site between days 14–21 following transplantation. The final tumour weight on death ranged between 8–11 g, 2·5–3·5 cm dia. Within the group of 20 untreated control animals, 3 mice developed small solid tumours (1–2 mm dia) subcutaneously at the injection site. Normal proliferation of Sarcoma 180 cells, in the ascitic form, within the peritoneal cavity of the untreated mouse far outstrips that of tumour cells growing subcutaneously as a solid. Such animals invariably die with gross ascites containing up to 5×10^8 cells, 16–18 days following the transplantation of 1×10^6 viable tumour cells. The incidence of solid tumours in mice transplanted with the ascitic form of Sarcoma 180 may be related to the time at which drug injections were given (days 3–7) and the total number of injections (5) received per treated mouse. 15% (3/20) of the untreated control animals developed small solid tumours at the site of the single injection received at transplantation. Whereas 69% (31/45) of the treated animals produced solid tumour at the injection site(s). It appears likely that an influx of a mixture of viable and non-viable tumour cells, from the ascitic fluid, may occur in the small lesion in the abdominal wall caused by the withdrawal of the hypodermic needle. The probability of development of a subsequent solid tumour at this site will then depend on the degree of invasiveness of the tumour and the amount of cytotoxic drug reaching the site.

The *in vivo* carcinostatic activity of the HPE-cysteine adduct, compared to HPE alone, against Sarcoma 180 in CBA/CA mice is shown in Fig. 4. For the HPE–cysteine adduct, the plotted dose is that dose of HPE which is assumed to be released on the breakdown with time of the adduct. For any given dose, in excess of 32 mg/kg body wt./day, treatment with HPE-cysteine i.p. is between 2–3 times more effective than HPE given as the free aldehyde.

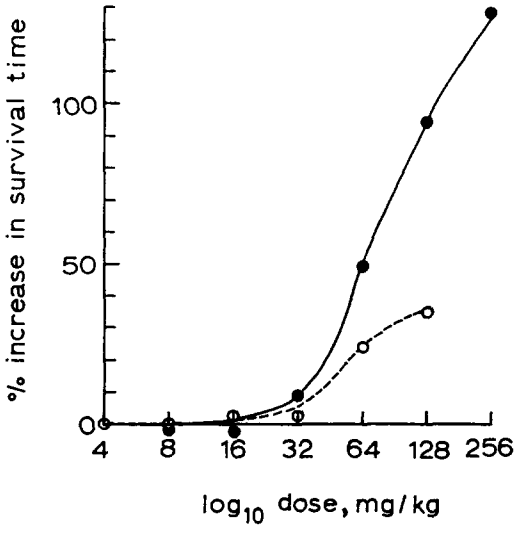


Fig. 4. Carcinostatic activity of a HPE-cysteine adduct in vivo *compared to HPE alone against Sarcoma* 180 *in female CBA/CA mice* (20–25 g). 1×10^6 *cells injected per mouse. The* % *increase in survival time, compared to untreated control animals, is plotted against* $\log_{10}$ *dose in mg/kg body wt./day;* O – – – O *HPE alone given on days* 3 *and* 4 *following transplantation in* 4 *divided doses* [1]*;* ●—●*, HPE–cysteine given on days* 3–7 *following transplantation in single doses.*

The results obtained are consistent with the view that the extended breakdown of an adduct of HPE and cysteine, releasing the reactive aldehyde, increases the half-life of HPE *in vivo*.

4. DISCUSSION

In this study, we have demonstrated that the incorporation of ^{3}H-thymidine into DNA of Sarcoma 180 tumour cells *in vitro* appears to be primarily affected, in a sigmoidal manner, at doses of HPE in excess of 0·16 mM. ^{3}H-uridine incorporation into RNA and ^{3}H-leucine incorporation into protein were considerably less depressed and declined linearly with increasing doses of added aldehyde *in vitro*. Therefore the mechanism of action of HPE against Sarcoma 180 and other tumours tested [3] in primarily depressing labelled thymidine incorporation into DNA *in vitro* appears to differ from that of most other aliphatic aldehydes which primarily depress protein synthesis *in vitro* [15].

The doses of added HPE required to reduce labelled thymidine incorporation into Sarcoma

180 cells *in vitro* by 50% (ID_{50}) and 90% (ID_{90}) were 0·09 mM and 0·157 mM respectively. We can compare those values with those obtained by Klamerth [18] on the depression of labelled thymidine incorporation produced by exposure for 5 hr of human fibroblasts in culture to increasing doses of malonaldehyde *in vitro*. HPE is approx. 7 times more effective than malonaldehyde on a molar basis in depressing thymidine incorporation into DNA over a considerably shorter exposure period (30 min compared to 5 hr). A small degree of protection against the effects of added aldehyde was obtained by the addition of 0·16 mM cysteine before preincubation of Sarcoma 180 cell suspensions with HPE *in vitro*. Preincubation of tumour cell suspensions with a premixed solution of cysteine and HPE was 2–3 times less effective, on a molar basis, than preincubation with HPE alone on the subsequent incorporation of ^{3}H-thymidine into DNA, compared to solvent treated control cultures. The results with HPE-cysteine (purified by TLC) indicated that a 1 hr preincubation of Sarcoma 180 cell suspensions was approx. 7 times less effective on a molar basis than treatment with with HPE alone *in vitro*. A 2 hr preincubation period in the presence of the adduct before the addition of labelled thymidine was approx. 4 times less effective than treatment with aldehyde alone. These results suggest that the HPE-cystine adduct is breaking down in the tissue culture medium, releasing 2 moles of cysteine for every mole of HPE, thus increasing the $t_{\frac{1}{2}}$ of HPE *in vitro*.

The effect of treating mice bearing the ascitic form of Sarcoma 180 with increasing doses of the HPE-cysteine adduct resulted in a series of dose-dependent increases in survival time, compared to untreated control animals. Within the group of tumour bearing animals treated at 128 and 256 mg/kg body wt./day of the adduct (expressed as mg of HPE assumed to be released) only 1 mouse in 20 had a mixture of ascitic fluid and a solid tumour present on death. The remainder (19 in 20) died with a large proliferated solid tumour (8–11 g. in wt., 2·5–3·5 cm in diameter) and the absence of ascitic fluid on death. It is significant that such solid tumours initially developed between days 14–21 following transplantation, at least 7 days after the last injection of HPE-cysteine. The initial development of these small solid tumours was correlated with the dose of the HPE-cysteine adduct received by the treated animals. Mice receiving 256 mg/kg body wt./day developed solid tumours later than those receiving smaller doses of the adduct. It appears likely that the concentration of cysteine and HPE circulating in the blood plasma following an i.p. injection of HPE–cysteine will be rather low. Certainly the normal detoxicification functions of the liver and other normal tissues [19] will ensure that within a few hours significant quantities will not be detected in the plasma following an i.p. injection of the adduct.

Treatment of mice implanted i.p. with the ascitic form of Sarcoma 180 with single daily injections i.p. of HPE-cysteine was 2–3 times more effective on a dose for dose basis than treatment of such animals with multiple daily injections i.p. of HPE alone [1]. The greatly lowered toxicity of the HPE-cysteine adduct compared to the free aldehyde injected i.p. has markedly improved the carcinostatic activity of HPE *in vivo*.

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Effect of Hyperthermia on the Radiation Response of two Mammalian Cell Lines*

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Abstract—*The interaction of* 43°C *hyperthermia and X-irradiation was compared* in vitro *in two cell lines: Chinese hamster ovary (HA-*1*) and mouse mammary sarcoma (EMT-*6*). In HA-*1 *cells the sequence of heating followed by X-irradiation resulted in appreciably more sensitization than the opposite sequence; in EMT-*6 *cells the opposite occurred. When time at* 37°C *was introduced between the two treatments, the degree of interaction was reduced, depending upon sequence and the duration of the* 37°C *time interval. In both cell lines, maximum toxicity was seen if the two treatments followed each other immediately.*

INTRODUCTION

THERE IS considerable evidence in the literature suggesting that elevated temperatures may be used as either the primary mode of tumor eradication or as an adjunct to other treatments; a review by Overgaard and Overgaard lists 131 references [1]. In order to develop methods for rational application, quantitative data on the effect of elevated temperatures on cells *in vitro* and *in vivo* as well as on tumors and normal tissues are required. Furthermore, in combined modalities, the optimal sequence of heat application and radiation or drugs must be evaluated.

Survival curves (surviving fractions vs duration of heat treatment) have been obtained for cells *in vitro*. These curves have a shape resembling that of X-ray survival curves [2, 3], although some observations indicate that heat survival curves have a resistant "tail" [4–6]. Cell inactivation by hyperthermia has been found to vary with position in the cell cycle [2–4], to depend on nutritional conditions and to some extent on the oxygen concentration [3–5, 7, 8]. Synergism is sometimes observed when cells are treated with drugs at an elevated temperature [9, 10], and when hyperthermia is combined with radiation [11–13]. For example, synchronous Chinese hamster cells were most sensitive when hyperthermic treatment (45·5°C for 7 or 11 min) was applied before irradiation [12]. In contrast, survival curves of V79 cells irradiated at 0°C with or without incubation at 42°C for 2 hr are virtually identical [13], and the degree of sensitization of Chinese hamster lung cells is approximately the same when thermal treatment at 42°C for up to 2 hr was applied either before or after irradiation [11]. It appears that the effect of hyperthermia on the radiation response depends on cell line, the temperature and duration of heat shock employed as well as on the sequence of the hyperthermia and irradiation.

We report here the effect of hyperthermia on the radiation sensitivity of a strain of Chinese hamster cells (HA-1), and on cells derived from a mouse mammary sarcoma (EMT-6). Specifically we compare the effects of ordering the X-irradiation and hyperthermia, and the effect of elapsed time between the two treatments.

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MATERIAL AND METHODS

Cells and culture conditions

One of the cell lines used was an established Chinese hamster ovary line, HA-1 [14]. The cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 15% fetal calf serum, penicillin and strepto-

mycin. Plating efficiencies in the experiments reported here were between 80% and 90%.

The other cell line was derived from a mouse mammary sarcoma, EMT-6 [15]. These cells were grown in Waymouth's medium supplemented with 15% fetal calf serum and antibiotics. Plating efficiencies with EMT-6 cells were usually between 50 and 70%.

Survival experiments

Two types of procedures were compared:

A. *Experiments on single cells.* Cells were trypsinized from exponentially growing cultures, counted and plated in 60 mm Petri dishes with fresh medium. The cells were incubated at 37°C for 3–4 hr to allow cells to attach to the plastic dish, and then were exposed to heat and/or X-ray irradiation. Afterwards, they were placed in an incubator at 37°C for 10 days without any additional manipulation to allow colony formation.

B. *Experiments on monolayers.* Exponentially growing cells were obtained by seeding 2×10^5 cells into 60 mm Petri dishes. Cultures were heated and/or irradiated at a cell density of approximately 10^5 cells/cm^2. After treatment, cells were trypsinized, counted and plated for colony formation.

Irradiation

Cells were irradiated at room temperature on a rotating platform. The X-ray parameters were 85 kvp, 9·6 mA, 1·5 mm Al-filter at an average dose rate of 130 rad/min.

Heating

Single cells or monolayers of cells on plastic Petri dishes were exposed to 43°C in specially designed hot water baths in incubators [5]. The dishes were placed on perforated trays so that the bottoms of the dishes were always in contact with the hot water. The temperature was maintained to ± 0·1°C and the pH of approximately 7·2 was maintained by a regulated gas flow of a mixture of 5% CO_2 and 95% air.

Definition and notation

We define the sequence of X-ray followed by 43°C temperature as postheat and the reverse as preheat. We use the following notation describing postheating experiments: dose + 43°C/t_H means the cells were irradiated with a dose of X-ray and then heated at 43°C for t_H min. Similarly, preheat experiments are described by 43°C/t_H + dose. The interaction experiments, i.e. experiments in which a sojourn at 37°C intervenes between the two

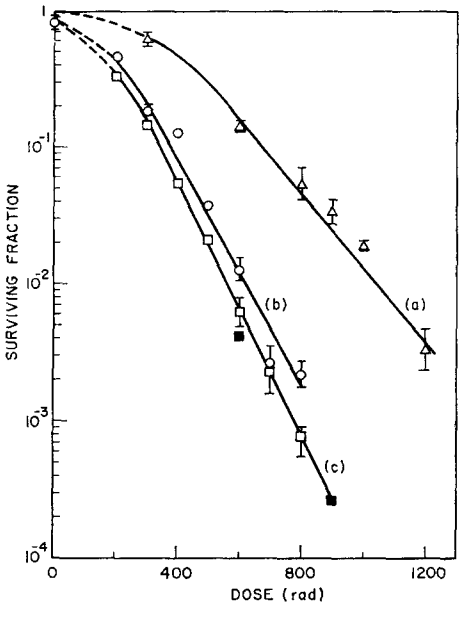

Fig. 1a. *HA-1 cells in Eagles MEM (with 15% FBS). (a) X-ray alone; (b) X-ray + 43°C/60 min; (c) 43°C/60 min + X-ray.*

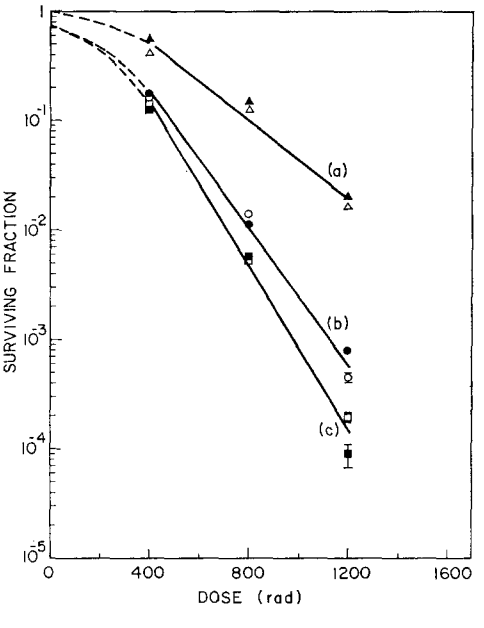

Fig. 1b. *EMT-6 cells in Waymouth medium (with 15% FBS). (a) X-ray alone; (b) 43°C/60 min + X-ray; (c) X-ray + 43°C/60 min.*

Fig. 1. *Dose–response curves of exponentially growing HA-1 and EMT-6 cells after sequential treatment of X-ray and 43°C heating. Closed triangles, squares and circles represent data points obtained from experiments on single cells, open triangles, squares and circles represent data points obtained from experiments on monolayer cells (see Material and Methods). The lines in the linear part of the survival curves are fitted to the data point according to the least squares method. Data points with bars represent mean values and the range of measured values obtained in at least two experiments.*

treatments, are described according to dose + 37°C/T_I+43°C/t_H (postheat) and 43°C/t_H+37°C/T_I+dose (preheat), thus T_I is the time (hr) at 37°C.

RESULTS

X-ray response

Survival values for HA-1 cells and EMT-6 cells exposed to graded dose of X-rays are shown in Fig. 1. The results are in good agreement with previously published data [16], and are used as controls for the experiments.

No statistically significant differences between survival values for cells which were treated either as single cells or monolayer cultures were observed. These results were observed for both HA-1 cells and EMT-6 cells, and in all experiments described in the text.

Heat response

The fraction of cells surviving heat exposure to 43°C for 60 min can be read from Fig. 1 (zero X-ray dose), and is approximately 80% for both cell lines.

Preheat response

Cells were exposed at 43°C for 60 min, then irradiated with graded doses of X-rays. Survival values immediately after the combined treatment of 43°C/60 min + dose are shown in Fig. 1a and Fig. 1b for HA-1 cells and EMT-6 cells respectively. The toxicity immediately after 43°C preheating and X-rays was more than additive. If it is assumed that the two types of damage (43°C and X-rays) are independent, then the cell survival for HA-1 cells after 43°C/60 min + 800 rad, should have been $\sim 1{\cdot}2 \times 10^{-1}$. However, a survival value of 5×10^{-3} was observed. Similarly, the survival for EMT-6 cells after 43°C/60 min + 800 rad should have been 4×10^{-2}; however, a survival value of 2×10^{-3} was observed. The almost 24-fold reduction in the survival of HA-1 cells (20-fold reduction for EMT-6 cells) implies an interaction between heat and X-ray damage and this is consistent with the observation of others [6, 11, 12, 13, 17]. As indicated by the different slopes of the survival curves in Fig. 1, the amount of reduction in survival values after preheating is dose-dependent.

Postheat response

Figure 1 also shows survival curves of cells which were irradiated with graded doses of X-ray and then exposed to 43°C for 60 min. If the assay was performed immediately after X-ray and postheating, then cell toxicity was again more than additive. A slightly lower

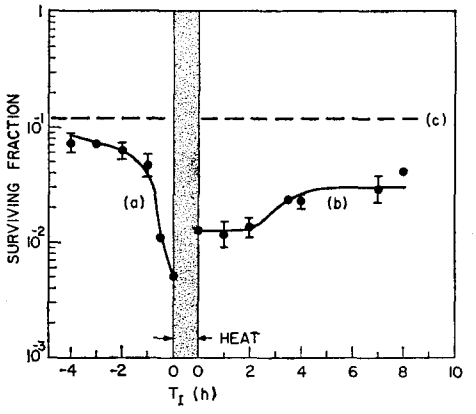

Fig. 2a. *HA-1 cells in Eagles MEM* (15% *FBS*).
(*a*) 600 *rad*+37°*C*/T_I+43°*C*/60 *min;*
(*b*) 43°*C*/60 *min*+37°*C*/T_I+600 *rad.*
(*c*) *Expected survival in the absence of interaction.*

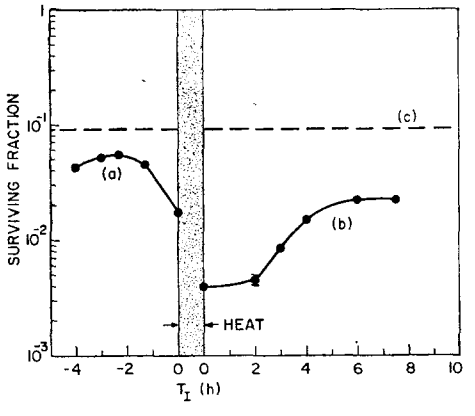

Fig. 2b. *EMT-6 cells in Waymouth medium* (15% *FBS*).
(*a*) 600 *rad*+37°*C*/T_I+43°*C*/60 *min;*
(*b*) 43°*C*/60 *min*+37°*C*/T_I+600 *rad.*
(*c*) *Expected survival in the absence of interaction.*

Fig. 2. *Interaction between X-ray and* 43°*C hyperthermia in exponentially growing HA-1 and EMT-6 cells.*

survival value was seen for EMT-6 cells immediately after the combined treatment dose + 43°C/60 min as compared to preheating, while a higher survival value for HA-1 cells was noted immediately after the combined postheating treatment dose + 43°C/60 min as compared to preheating. In all cases the amount of reduction in survival values was dose-dependent.

Effect of time (37°*C*) *between treatments*

Cells were given 600 rad, afterwards either immediately exposed to 43°C for 60 min or incubated at 37°C for 1–4 hr, and then exposed to 43°C for 60 min. The surviving fractions for 600 rad + 37°C/T_I + 43°C/60 min are shown in Fig. 2a and Fig. 2b for HA-1 cells and EMT-6 cells respectively. Similar experiments between 43°C preheating and X-ray were also performed. Cells were exposed to

43°C for 60 min, afterwards either exposed to 600 rad immediately or incubated at 37°C for 1–8 hr and then exposed to 600 rad of X-ray. The resulting surviving fractions for 43°C/60 min + 37°C/T_I + 600 rad are shown in Fig. 2a and Fig. 2b for HA-1 and EMT-6 cells respectively. In these experiments the amount of radiation given was the same and the amount of heating was also identical; the only two variables were the time interval (37°C/T_I) between the radiation and heat exposure and the sequence of the treatments.

What is of interest here is the comparison between survival values predicted on the basis of additivity of 43°C and X-ray (curves C in Fig. 2a and 2b) and the actual measurements. In the postheating experiments, there was a 5-fold difference for HA-1 cells and a 20-fold difference for EMT-6 cells if 43°C exposure was immediately followed by X-ray, and a 2-fold difference for both HA-1 and EMT-6 cells if heating was delayed by 2 hr (T_I = 2 hr). This 2-fold difference remained constant even if the heating was delayed by 4 hr (T_I = 4 hr).

In the preheating experiments there was a 20-fold difference for HA-1 cells and a 10-fold difference for EMT-6 cells if irradiation followed immediately after heating, but only a 5-fold difference for both HA-1 and EMT-6 cells if X-ray was delayed by 4 hr (T_I = 4 hr). This 5-fold reduction in survival remained unchanged even if irradiation was delayed by 8 hr for both cell lines.

DISCUSSION

Treatment of cells as either single cells or in monolayer cultures resulted in identical surviving fractions (within statistical fluctuations). This is observed for both EMT-6 and HA-1 cell lines, and it indicates that any possible interference of trypsin with repair processes, if such interference exists at all, has very little effect on cell survival. Furthermore, cell to cell contact at the time of heating appears not to be of importance.

For HA-1 cells, there is greater initial sensitization in the case of preheating than postheating, while for EMT-6 cells greater initial sensitization is observed in the case of postheating. We cannot offer any explanation for this difference between the two cell lines.

Our results confirm that X-ray and 43°C postheating interact in the sense that the combined treatment is more cytotoxic than the sum of the individual treatments. The maximum toxicity occurs when heat follows immediately after X-ray. This irradiation-induced heat sensitivity then is reduced as the two treatments are separated by time interval at 37°C. However, survival values do not reach those calculated on the basis of additivity, even if the time at 37°C exceeds 4 hr. Somewhat similar results are observed in plateau phase HA-1 cells [17], although there once the repair of X-ray induced damage was completed ($T_I \geqq 4$ hr), the cells surviving the X-irradiation responded to 43°C heat shock as if they had never been exposed to X-ray. For exponentially growing HA-1 and EMT-6 cells, the difference in surviving fractions between predicted value (based on simple additivity) and actual measurements for $T_I \geqq 4$ hr can be explained on the basis of differences in the age response of cells to X-ray and hyperthermia, and possibly the X-ray induced para-synchronization of the cell population. Westra and Dewey [2] have shown that X-ray resistant late S phase cells are particularly sensitive to hyperthermia.

Preheating and X-irradiation also interact. Again maximum toxicity is observed when the two treatments immediately follow each other. As T_I increases, the survival increases. At $T_I \geqq 4$ hr it reaches 25% of the value predicted on the basis of additivity. The rate of increase in surviving fraction is only half the rate seen for postheating. It seems that heat introduces a transitory state during which cells are radiation sensitive. This state decays gradually and this represents a partial recovery from heat induced damage. In an earlier paper [18] it was reported that after a 1 hr heat exposure at 43°C cells accumulate in the S phase during the first 4–6 hr after heat treatment and subsequently accumulated in G_2+M. For both HA-1 and EMT-6 lines, cells in late S as well as in early G_1 are radio-resistent, cells in late G_1, early S and G_2 + M are radio-sensitive [19]. The 1-hr and 2-hr points of Fig. 2 (preheating) represent surviving fractions of populations with relatively large fractions of cells in late G_1 and early S. These are relatively radiosensitive, however, for the 4–8 hr points, relatively large fractions of cells are accumulated in late S and these are radio-resistant. Consequently the increase in survival may well be explained by a competition of this para-synchronization with a recovery process from heat induced X-ray sensitivity and the variation in sensitivity of cells to heat over the cell cycle.

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Role of Fetal Antigens in Tumor Immunity

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Abstract—*Studies were undertaken to demonstrate the role of fetal antigens in tumor immunity. Viable fetal cells inoculation followed by challenge with transplanted methylcholanthrene induced tumor cells after* 7, 14 *and* 21 *days significantly delayed the time of appearance of tumors. Transfer of lymphoid cells from mice immunized with fetal cells followed by tumor challenge resulted in delayed appearance of tumors. Inoculation of viable tumor cells pre-treated* in vitro *with serum from mice immunized with fetal cells shortened the time of appearance of tumors in mice.*

INTRODUCTION

THE DEMONSTRATION of tumor specific transplantation antigens in tumors and the demonstration of both humoral and cell-mediated immunity to these antigens have opened up great possibilities of immunologic control of cancer [1, 2]. Several workers from time to time have suggested that animal and human tumor antigens are, in fact, reexpressed fetal components which appear in early fetal life [3–6].

However, the role of these tumor-associated fetal antigens in tumor rejection response remains controversial. Prehn [7], Lemevel and Wells [8], Bendich *et al.* [9] reported protection of C57B1/6 and C57 BL mice against methylcholanthrene-induced tumors by pre-immunizing with embryo cells or by exposure to fetal antigens during pregnancy. Grant *et al.* [10] also reported significant protective effect to chemically induced tumor challenge following immunization with fetal tissue. However, Blair [11], Castro *et al.* [12], Baldwin *et al.* [13], Parmiani and Lembo [14] reported that immunization with fetal tissue does not elicit resistance to challenge with chemically induced hepatomas and sarcomas. Brawn [15], Girardi *et al.* [16]. Grant *et al.* [10] showed that multiparous female animals were resistant to tumor challenge and they possessed cytotoxic lymph node cells that destroyed several viral and methylcholanthrene-induced tumor cells *in vitro* and *in vivo*. By means of blocking antibody studies, Baldwin *et al.* [6, 13], Hellstrom and Hellstrom [17, 18] demonstrated that serum from multiparous rats blocked the cytotoxicity of lymph node cells from multiparous rats both to tumour cells as well as to fetal cells.

The present studies were undertaken to explore the possible immunological similarity between the antigens of fetus and methylcholanthrene-induced fibrosarcoma in inbred mice of C57 strain. The effect of fetal cells inoculation followed by tumor challenge at different intervals, the effect of lymphoid cells and sera from mice immunized with fetal cells and the effect of lymphoid cell transfer from multiparous pregnant mice on the time of appearance of tumors was studied in different groups and compared with appropriate control groups.

MATERIAL AND METHODS

Animals

All animals used belonged to an inbred strain C57 (Jet black, ICRC) mice maintained by of continuous single line brother to sister mating. The purity of inbred lines was tested routinely by acceptance of the reciprocal skin grafts.

Tumors

Primary fibrosarcomas were produced by 20-methylcholanthrene (MC) according to the method of Reiner and Southam [19]. Tumors were then routinely passaged by subcutaneous

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transplantation in syngeneic mice. Inoculation of C57 inbred mice with 1×10^6 viable tumor cells has always given 100% tumor incidence in a large group of animals challenged. The tumors become palpable within 8–10 days. All experiments were conducted on passaged tumors. The demonstration of transplantation immunity in syngeneic animals after surgical removal of the tumor was in support of the fact that this tumor is antigenic.

Cell viability test

Viability of cells was tested by trypan blue dye exclusion test.

Immunization with fetal cells

First litters from 12 week old C57 mice were used as a source of fetal tissue. Timed matings were achieved and the number of vaginal plugs was recorded. The day the plug was recorded was considered day 0. Embryos 8–10 days old, from primiparous female mice, were removed under aseptic conditions, dissected free of placenta and membranes with sharp forceps and were placed in cold Eagle's minimum essential medium (MEM). The fetuses were then homogenized gently in glass homogenizer. Cells were sieved through a sterile stainless steel (80 mesh) gauze and washed twice in MEM. Trypan blue dye exclusion test was performed for cell viability, 2×10^8 viable cells/0·1 ml were then inoculated under the kidney capsule of adult male mice, with a very fine needle. These mice were then divided into five groups. Mice of these different groups were then challenged with 1×10^6 viable tumor cells at interval of 0, 7, 14, 21 and 28 days respectively by subcutaneous route. Simultaneously, a control group was challenged with tumor cells only. In another set of experiments the mice were inoculated with 2×10^8/0·1 ml normal adult spleen cells under the kidney capsule and challenged at different intervals with 1×10^6 viable tumor cells. The initial time of appearance of tumors in mice of different groups was recorded and statistically compared. In addition, unchallenged recipients injected with fetal cells were sacrificed at intervals to study the histology of the growing fetal tissues under the kidney capsule.

Lymphoid cell transfer

To study the effect of embryonic antigen sensitized lymphoid cells, lymph node cell suspensions were prepared separately from mice of following three groups:

Group I: Normal virgin mice.

Group II: Mice immunized with fetal cells (after 8 days of immunization).

Group III: Multiparous pregnant mice previously having had more than 4 pregnancies.

The cell suspensions were passed through 80 mesh stainless steel screen and washed twice in BSS. Cells were finally suspended in MEM.

Normal adult male mice were divided into three groups and each mouse of each group received two doses of 4×10^8/0·5 ml lymph-node cells from mice of above groups respectively at four days interval by intra-peritoneal route. After 7 days of last transfer, these mice were challenged with 1×10^6 viable tumor cells. Initial time of appearance of tumors in all mice was noted and compared.

Inoculation of tumor cells treated with serum

To study the effect of serum from fetal cells immunized mice, normal syngeneic mice were injected subcutaneously in three different groups with 1×10^5 viable tumor cells/mouse incubated previously for 30 min. at 37°C with 0·5 ml of MEM medium, with 0·5 ml of normal virgin mice serum and with 0·5 ml of serum from mice immunized with fetal cells respectively. These mice were then observed to record the initial time of appearance of tumors. Serum was collected after 28 days of fetal cells injection.

RESULTS

Histological studies

Normal adult male mice were inoculated under the kidney capsule with cells from syngeneic 8–10 days old fetuses. These mice were then divided in 4 groups of 4 mice in each group and sacrificed at intervals of 7 days, 14 days and 28 days. Their kidneys were examined macroscopically and microscopically. Figure 1 shows a mouse kidney with a fetal growth on its surface. Kidney sections from mice killed after seven days of fetal cells inoculation revealed proliferation of undifferentiated cells under capsule of kidney (Fig. 2). After fourteen days of fetal cells injection, there was beginning of formation of more organized structures with cavity formation (Fig. 3). Kidney sections from mice sacrificed after 28 days of fetal cells inoculation, showed the development of teratomatous elements consisting of mature bone with bone marrow cells, cavities lined by columnar epithelium, cartilage and undifferentiated cells showing more organized pattern of development (Fig. 4).

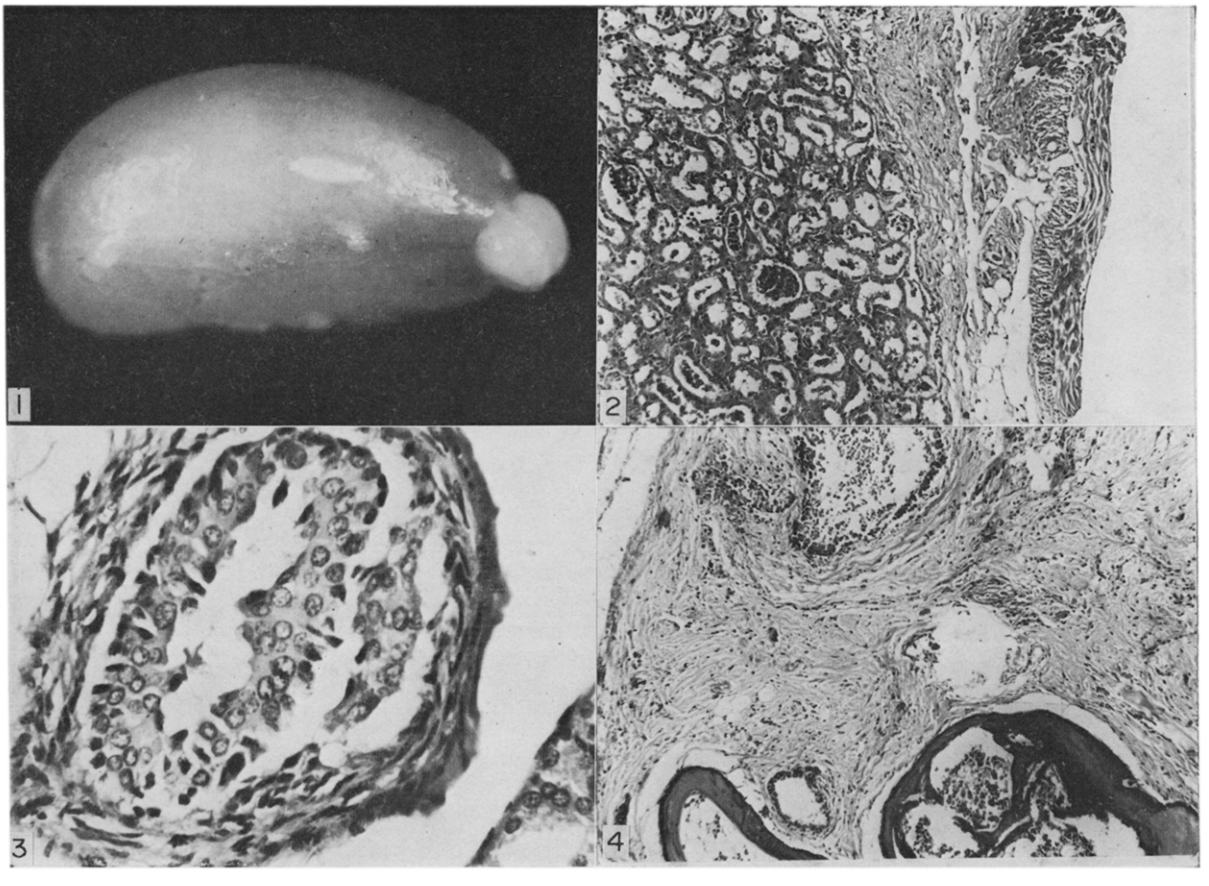

Fig. 1. *A mouse kidney showing a subcapsular growth of fetal cells.*

Fig. 2. *Microphotograph of a kidney section from mouse sacrificed after* 7 *days of fetal cell inoculation showing proliferation of undifferentiated cells under kidney capsule* (*H and E* × 110).

Fig. 3. *Microphotograph of a kidney section of mouse sacrificed after* 14 *days of fetal cell inoculation showing cavity formation* (*H and E* × 440).

Fig. 4. *Microphotograph of a kidney section of mouse sacrificed after* 28 *days of fetal cell inoculation showing a mature bone with bone marrow cells, cavities lined by columnar epithelium, cartilage and undifferentiated cells* (*H and E* × 110).

Effect of fetal cell inoculation

Figure 5 compares the time of appearance of tumors in mice injected with fetal cells followed by tumor challenge at different intervals. Mean time of appearance of tumors in control mice was $8{\cdot}62 \pm 0{\cdot}93$ and in mice inoculated with fetal cells followed by tumor challenge after 7, 14 and 21 days, the mean time of appearance of tumors was $22{\cdot}9 \pm 5{\cdot}17$ days, $17{\cdot}6 \pm 3{\cdot}75$ days and $15{\cdot}1 \pm 2{\cdot}68$ days respectively. These mean values are significantly different when compared to those of control mice ($P < 0{\cdot}05$). The mean time of appearance of tumors in mice challenged on 0 day and after 28 days of fetal cell inoculation was $10{\cdot}5 \pm 2{\cdot}14$ and $10{\cdot}8 \pm 1{\cdot}76$ days respectively. These values are not significantly different from the control mice ($P > 0{\cdot}05$). The mean time of tumor appearance in mice inoculated with normal adult spleen cells followed by tumor challenge after 0, 7, 14, 21 and 28 days was $8{\cdot}25 \pm 0{\cdot}78$ days, $9{\cdot}25 \pm 1{\cdot}03$ days, $9{\cdot}0 \pm 1{\cdot}32$ days, $8{\cdot}75 \pm 1{\cdot}03$ days and $9{\cdot}0 \pm 1{\cdot}32$ days respectively. These values are not significantly different as compared to that of control mice ($P > 0{\cdot}05$). Except for 0 and 28 days, all these mean values are significantly different ($P < 0{\cdot}05$) as compared to the mean values of corresponding groups of mice inoculated with fetal cells under the kidney capsule.

Effect of lymphoid cells from fetal cells immunized from multiparous mice

Figure 6 compares the effect of lymphoid cells from fetal cells inoculated mice and from multiparous pregnant mice for their capacity to adoptively transfer resistance to tumor challenge. Mean time of appearance of tumors in control mice challenged with tumor cells only was $9{\cdot}4 \pm 1{\cdot}35$ days. Normal lymphoid cells did not affect the time of appearance of tumors since the mean time of appearance of tumors in these mice was $9{\cdot}6 \pm 2{\cdot}33$ days. Mean time of tumor appearance in mice transferred with lymphoid cells from fetal cells immunized mice and from multiparous pregnant mice was $17{\cdot}2 \pm 2{\cdot}4$ days and $14{\cdot}0 \pm 1{\cdot}76$ respectively. These mean values are significantly different as compared to both the control groups ($P < 0{\cdot}05$).

Effect of serum

The results of this experiment are presented in Fig. 7. Pre-treatment of tumor cells with serum from fetal cells injected mice did not delay the tumor appearance. On the other hand, it shortened the time of appearance of tumors. The mean time of appearance of tumors in mice injected with tumor cells treated with serum from fetal cells immunized mice was $14{\cdot}0 \pm 1{\cdot}5$ days and in mice injected with normal serum treated tumor cells and MEM medium treated tumor cells was $18{\cdot}25 \pm 1{\cdot}7$ days and $19{\cdot}25 \pm 1{\cdot}3$ days respectively. These mean values are significantly different as compared to that of experimental animals ($P < 0{\cdot}05$).

DISCUSSION

There is considerable evidence to suggest the existence of a variety of fetal antigens in association with human tumors [3, 4, 20, 21]. Experimental studies have further proved the association of embryonic antigen with chemically induced tumors [6, 22, 23]. Tumors induced by viruses have also been shown to share antigens with embryos [24, 25].

Prehn [7] for the first time found suppression of transplanted MC induced tumor growth in mice pre-immunized with viable embryo cells. Blair [11], Ting *et al.* [26], Castro *et al.* [12], Baldwin *et al.* [13], Parmiani and Lembo [14] failed to find transplantation immunity against virus induced mammary tumors, SV40 induced tumors and chemically induced tumors after immunization with fetal cells. But studies of Buttle and Frayn [27], Coggin *et al.* [24], Girardi *et al.* [16] Lemevel and Wells [8], Bendich *et al.* [9] and Grant *et al.* [10] encouraged various workers to carry on further studies with other tumor types since these workers showed that preimmunization with fetal antigens produced significant resistance against development and suppression in growth of imferon induced sarcoma, SV40 induced tumors, adeno virus induced tumors, Rouscher leukemia virus injected cells, plasma cell tumors and MC induced tumors.

In the present studies, inoculation of fetal cells followed by tumor challenge after 7, 14 and 21 days resulted in significant delay in tumor appearance as compared to the control mice. Tumor challenge on 0 day and after 28 days of fetal cells inoculation did not affect the time of appearance of tumors (Fig. 5).

Failure of different investigators to induce immunity against various types of tumors following immunization with fetal cells may be attributed to several factors and different experimental conditions. First and most important may be the strain dependent sensitivity. Whether the host responds to these fetal antigens or not will determine their suitability for immunotherapy. Ting *et al.* [25] could produce anti fetal serum in C57 mice which was found to be highly reactive. Antisera

produced by the same schedule in C3H mice was weakly reactive whereas repeated hyper-immunization of BALB/C or CBFI mice with X irradiated fetal cells failed to produce serum that would react with tumor antigen or fetal antigen. Several workers reported the induction of immunity against tumors in C57 mice by preimmunizing them with fetal cells [8, 9], while Blair [11], Ting *et al.* [26], Parmiani and Lembo [14] failed to induce such immunity in BALB/C mice. Similarly hamsters have been found to be good responders to fetal antigen as compared to BALB/C mice [25, 10].

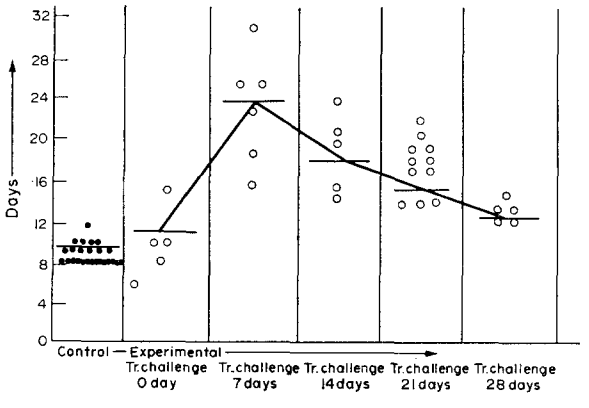

Fig. 5. *Figure showing the effect of fetal cells inoculation followed by tumor challenge at different intervals on time of appearance of tumors in mice.*

Whether sex plays any significant role in response to fetal antigens is again a controversial question. Some workers found that males responded better to fetal antigen immunization [24, 16]. However, Grant *et al.* [10] did not find any such sex difference in case of MC induced tumors.

The degree of immunity also depends upon the immunization schedule, latent period etc. In the present study, failure of viable fetal cells to induce resistance in mice challenged with tumor cells after 28 days may be explained on the basis that fetal tissues become mature in adult hormonal environment as has been discussed by Coggin *et al.* [24]. This is confirmed by the histological study of fetal cell growth below the kidney capsule in mice sacrificed after 28 days of fetal cell injection which constituted almost all mature structures like bone with bone marrow, cartilage, cavities lined by columnar epithelium (Fig. 4). However, it is difficult to suggest whether the tumor associated fetal antigens were present at this stage or not. Appearance of blocking antibodies by this time may be another factor. Buttle and Frayn [27] found that degree of tumor inhibition was highest if the interval between fetal cells injection and tumor challenge was 7 days and the effect gradually reduced as this interval was increased.

Under natural conditions only females are exposed to fetal antigens during pregnancy. Blair *et al.* [28] reported that spontaneous tumor development in the virgin females occurs at a later time than in the parous females. On the other hand, Fraumeni *et al.* [29] after studying a large group of patients concluded that unmarried females are more susceptible to tumors of the colon, breast, corpus uteri and ovary. Apart from hormonal influences, the cause of this difference may be that during pregnancy females are exposed to fetal antigens which sensitize the lymphoid cells. Baldwin *et al.* [5, 6], Steele and Sjogren [23], Hellstrom and Hellstrom [17, 18] reported that multiparous female animals possess cytotoxic lymph node cells that destroy several viral and MC induced tumors *in vitro*. Very few investigators have tried to demonstrate the *in vivo* role of fetal antigen sensitized lymphocytes in tumor rejection response. Only few contradictory reports are available. In the present investigation studies were undertaken to examine the capacity of lymphoid cells from fetal cells injected mice and from multiparous pregnant mice to transfer adoptively the resistance to tumor challenge. It was found that lymphoid cells from fetal cells immunized mice and from multiparous pregnant mice significantly delayed the time of appearance of tumors as compared to the control mice whereas the normal lymphoid cells did not (Fig. 6).

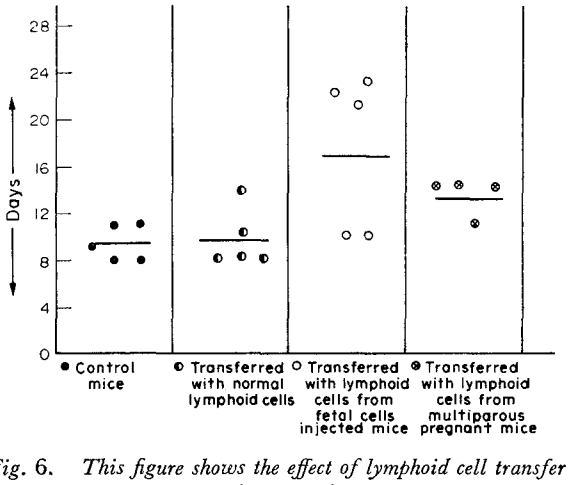

Fig. 6. *This figure shows the effect of lymphoid cell transfer from mice immunized with fetal cells and from multiparous pregnant mice on appearance of tumors.*

Hanna *et al.* [30] reported that transfer of spleen cells from multiparous mice protected the normal mice from infection with Rouse leukemia virus. Girardi *et al.* [16] showed that

lymphoid cells from primiparous and multiparous hamsters protected normal male hamsters against SV40 induced tumor challenge. On the other hand, studies of Baldwin *et al.* [13], Parmiani and Lembo [14] showed that lymphoid cells from multiparous rats and mice were ineffective in adoptively transferring tumor immunity to normal animals.

In the next set of experiments, three groups of normal mice received tumor cells incubated previously with MEM, with serum from normal virgin female mice, with serum from fetal cells immunized mice respectively. Inoculation of mice with tumor cells treated with serum from fetal cells immunized mice did not protect them against tumor formation (Fig. 7). Surprisingly, the tumor appearance time was shortened in these mice as compared to the control groups.

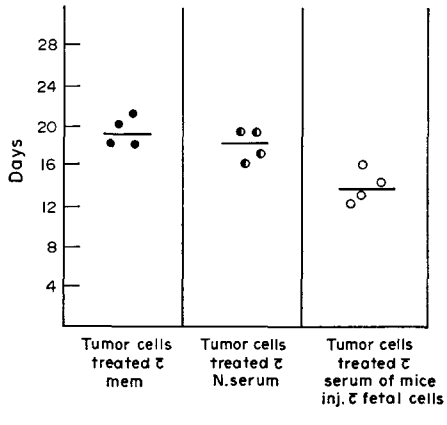

Fig. 7. This figure shows the effect of in vitro *treatment of tumor cells with serum from normal mice and from mice immunized with fetal cells on time of appearance of tumors.*

Coggin and Anderson [31] demonstrated that females splenectomized to reduce the antibody forming capacity react protectively to fetal vaccine. Antibody response against fetal antigens is more because these antigens contain more antigenic sites as compared to the tumor antigen. Recently in a series of their *in vitro* studies, Baldwin *et al.* [6, 13] have shown that lymph node cells from multiparous pregnant mice were cytotoxic *in vitro* for cultured tumor cells and fetal cells. But this cytotoxicity for tumor cells and fetal cells was blocked in presence of serum from multiparous mice (Fig. 8). Similar findings have been given by Hellstrom and Hellstrom [17, 18] who ascribed the inability of fetal antigens to induce tumor resistance in body to these blocking antibodies.

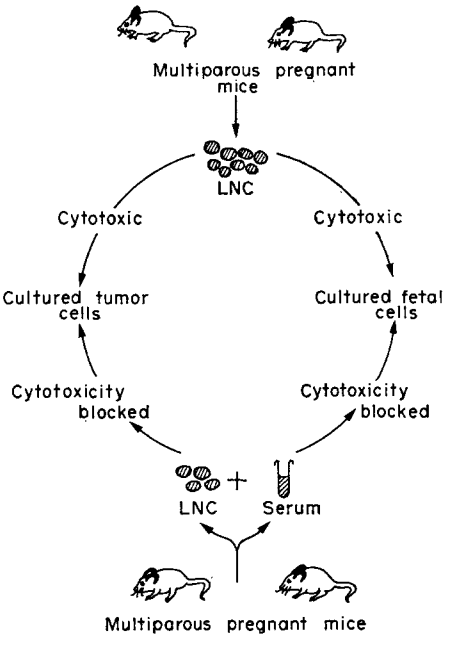

Fig. 8. Diagrammatic presentation of in vitro *cytotoxicity of lymphoid cells from multiparous mice against tumor cells, fetal cells and blocking of this cytotoxicity by serum from multiparous mice.*

Recently, Tamerius *et al.* [32] described that fetal antigens have a special property to form blocking factors. These factors prevent either the development of cell mediated immunity or act against the expression of an already developed cell mediated immunity.

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Prediction of Marrow Toxicity in Patients Treated by Intravenous Infusion of 5 Fluorouracil*

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Abstract—*Bone marrow aspirates from patients receiving intravenous infusions of* 5 *fluorouracil* (5*FU*)¶ *for gastrointestinal malignancies were tested by measuring the rate of incorporation of* ^{3}*H-UdR and* ^{3}*H-TdR into DNA in the presence of* 5*FU and the effect of* 5*FU on the ability of marrow cells to form granulocytic colonies* (*CFU-C*) *in methyl cellulose. The best correlation found was between toxicity and the ratio of the rate of* ^{3}*H-TdR and* ^{3}*H-UdR incorporation before infusion, after the addition of* 5*FU to the incubations. Seven of eight cases showed less inhibition of* ^{3}*H-UdR incorporation by* 5*FU after completing their infusion, representing a drug-induced resistance not apparently related to the proliferative state of the marrow. Two patients with the lowest peripheral granulocyte count relative to pre-treatment values also showed the greatest depression of granulocyte colony forming ability when their marrow cells were treated with* 5*FU at a concentration of* $1 \times 10^{-5} M$. *Although further cases need to be studied, these techniques appear to be of predictive value in determining marrow toxicity to* 5*FU.*

THE DRUG, 5 fluorouracil (5FU), produces less toxicity when given by intravenous infusion for five days than when given as a single intravenous injection [1, 2]. Nevertheless, mucositis occurred in all of 34 patients treated by infusion of drug for five days and leukopenia in four patients [2]. If such toxicity could be predicted, discomfort and danger to the patient could be avoided and dosage increased in those patients who do not show toxic effects. The aim of this study was to predict toxic effects of the drug on bone marrow by determining the sensitivity of DNA synthesis in bone marrow cells to 5FU added *in vitro*, before and after infusion. Synthesis of DNA was measured by the rate at which ^{3}H-TdR and ^{3}H-UdR were incorporated into acid insoluble material. The former is a measure of the rate of incorporation of TTP into DNA by the "salvage" pathway and the latter rate, incorporation of TTP by the "de novo" pathway which is specifically inhibited by 5FUdRP formed from 5FU [3]. As well, the number of CFU-C's formed after treating marrow cells with 5FU *in vitro* was compared to the number formed by untreated marrow cells.

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¶Abbreviations: 5FU, 5 fluorouracil; ^{3}H-UdR, tritium labelled deoxyuridine; ^{3}H-TdR, tritium labelled thymidine; TCA, trichloroacetic acid; TTP, thymidine triphosphate; 5FUdRP, fluorodeoxyuridylic acid; CFU-C, granulocyte colony forming units; UR, uridine.

MATERIAL AND METHODS

Patients

Patients with malignant disease of the gastrointestinal tract received intravenous infusions of 5FU for five days at a dosage of 30 mg/kg/day in 5 % dextrose at a rate of one litre per day. Peripheral blood counts were performed and the nadir of the granulocyte count expressed as a percentage of the granulocyte count before infusion. Platelet counts were little affected by the treatment and stomatitis, contrary to the

findings of Seifert *et al.* [2], was absent or mild in all cases.

Bone marrow samples

Bone marrow was aspirated before infusion of drug and within five hours of stopping the infusion. The number of nucleated cells were counted by means of a hemocytometer using the method described by Dacie and Lewis [4].

Incorporation of isotope

Bone marrow cells were washed, then resuspended in RPMI 1640 medium containing 10% fetal calf serum, Two ml of the suspension ($0{\cdot}5 \times 10^6$ to 3×10^6 cells) were dispensed in 10-ml conical flasks, gassed with 5% carbon dioxide and incubated at 37° in a shaking bath for 30 min. We then added 5FU to give a concentration of 1×10^{-5}M and incubated for 1 hr. ^{3}H-TdR or ^{3}H-UdR was then added to give a final concentration of 1×10^{-6}M and 1 μcurie per ml. After 30 minutes, we added 8 ml of 10% cold trichloroacetic acid (TCA) and cooled the flasks at 4°C for a further 30 min. The precipitate was washed twice with cold 10% TCA, dissolved in 1 ml of NCS and added to 10 ml of scintillation fluid for counting: duplicate or triplicate samples were used when enough cells were available. Results were calculated as dis/min per 10^6 nucleated cells and as a percentage of the untreated control.

Bone marrow culture

Buffy coats prepared from bone marrow aspirates obtained prior to treatment were washed once in 10 ml of complete alpha-medium (Flow Laboratories, Bethesda, Maryland). An incubation mixture consisting of 0·7 ml of cell suspension, 0·1 ml of 5FU diluted with incomplete alpha-medium and 0·2 ml of fetal calf serum (Grand Island Biological Company) was prepared and incubated at 37°C for 1 hr. In three cases, the nucleated bone marrow cells were exposed only to one drug concentration, 1×10^{-5}M. In all other cases, three drug concentrations, 1×10^{-5}M, 1×10^{-4}M and 1×10^{-3}M were used. The rationale for the use of incomplete alpha-medium (i.e. without nucleosides and deoxynucleosides) is discussed by Madoc-Jones *et al.* [5]. Following the incubation, the cells were washed once in 40–50 ml of incomplete alpha-medium, resuspended in complete alpha-medium and plated in methyl-cellulose according to the method of Iscove *et al.* [6]. The final cell concentration in the plating mixture varied from 1×10^4 to 2×10^5, depending on the number of available cells. Control cell samples (the patient's own cells) were similarly treated, except that the drug solution was omitted from the incubation mixture. The mixture was plated in 35 mm Petri Lux-dishes (Flow Laboratories) and the cells were allowed to grow in a moist atmosphere with 7·5% CO_2 and air. The number of CFU-C's present on day 14 of incubation was determined using an inverted microscope and the criteria of Messner *et al.* [7]. The number of colonies obtained from untreated cells ranged from 40 to 80 per 2×10^5 nucleated cells plated. This number was taken as 100%, while those obtained from cells treated with various drug concentrations were expressed as a percentage of those control values.

RESULTS

Tables 1 and 2 show that the rate of incorporation of ^{3}H-UdR into bone marrow cells vary from 1638 to 25,400 dis/min per 10^6 nucleated cells before treatment and in the absence of added 5FU. That for ^{3}H-TdR showed a similarly marked variation from 3262 to 64,800 dis/min per 10^6 nucleated cell. Such large variations probably represent an increased proliferative state of the nucleated cells in the marrows of patients one and seven. The remaining six patients show a much smaller variation, from 1638 to 6302 for ^{3}H-UdR and 3262 to 9569 for ^{3}H-TdR. The ratios of the rates of ^{3}H-TdR and ^{3}H-UdR incorporation, shown in Table 3, show less variation from 1·5 to 6·2 before treatment and without added 5FU. With added 5FU, however, the variation is much greater, from a ratio of 1·3 to 23·2. This table also shows that patients with the highest ratios had the most severe depression of circulating granulocytes after infusion of 5FU. Figures 1 and 2 confirm this and show a good correlation between the ratios of TdR and UdR rates before treatment and the percentage granulocytes at nadir rather than with the same ratio after infusion and/or with the absolute granulocyte number. One or two patients did not show this correlation.

Colony-forming units in vitro

Figure 3 shows a linear relationship between the number of cells plated and the number of granulocyte colonies grown from cells not exposed to drugs (control) and those exposed to 1×10^{-5}M concentration of 5FU. Only in two of the eight cell samples studied did this drug concentration produce a significant depression of surviving CFU-C. Figure 4 shows the dose-survival curves obtained by plotting on a semi-logarithm scale the percentage surviving CFU-C against four concentrations of 5FU, 1 ×

Table 1. *Effect of 5FU on ^{3}H-UdR Incorporation in Bone Marrow Cells before and after Treatment of Patients with 5FU*

Patient	Absolute granulocyte count	Percentage granulocyte count	UdR incorporation					
			Before treatment			After treatment		
1	150*	6%†	9150‡	3860§	(42%)	10,520‡	8870§	(83%)
2	1500	35%	1638	600	(64%)	3921	1493	(81%)
3	1350	43%	2005	1202	(68%)	3256	1255	(81%)
4	3700	52%	2413	1849	(77%)	1511	1221	(81%)
5	2400	67%	3333	2172	(65%)	2660	1984	(75%)
6	3500	68%	6302	2936	(40%)	13,767	8141	(59%)
7	6400	100%	25,400	13,170	(52%)	22,430	17,120	(76%)
8	6400	100%	3307	2214	(67%)	4389	3021	(67%)

*Nadir as granulocytes/cmm
†Nadir as % of original peripheral granulocyte count
‡dis/min per 10^6 nucleated cells
§dis/min after treatment with 1×10^{-5}M 5FU for 1·0 hr.

Table 2. *Effect of 5FU on ^{3}H-TdR Incorporation in Bone Marrow Cells before and after Treatment of Patients with 5FU*

Patient	Absolute granulocyte count	Percentage granulocyte count	TdR incorporation					
			Before treatment			After treatment		
1	150*	6%†	56,300‡	89,488§	(159%)	106,410‡	108,410§	(101%)
2	1500	35%	3262	3562	(109%)	12,585	11,164	(89%)
3	1350	43%	5978	6226	(104%)	7457	7169	(96%)
4	3700	52%	6365	6085	(96%)	4906	4975	(100%)
5	2400	67%	5367	5797	(108%)	7779	7577	(97%)
6	3500	68%	9569	3916	(41%)	61,009	72,568	(11%)
7	6400	100%	64,800	76,400	(118%)	39,410	47,880	(12%)
8	6400	100%	5556	3637	(65%)	9217	10,408	(113%)

*Nadir as granulocytes/cmm
†Nadir as % of original peripheral granulocyte count
‡dis/min per 10^6 nucleated cells
§dis/min after treatment with 1×10^{-5}M 5FU for 1·0 hr.

Table 3. *Ratios of rates of TdR and UdR incorporation before and after treatment with 5FU related to nadir of granulocyte counts*

Patient	Absolute granulocyte count	Percentage granulocyte count	Before treatment		After treatment	
			No added 5FU	Added 5FU	No added 5FU	Added 5FU
1	150*	6%†	6·2‡	23·2‡	10·1§	12·2§
2	1500	35%	2·0	5·9	3·2	7·5
3	1350	43%	3·0	5·2	2·3	5·7
4	3700	52%	2·6	3·3	3·2	4·1
5	2400	67%	1·6	2·7	2·9	3·8
6	3500	68%	1·5	1·3	4·4	8·9
7	6400	100%	2·6	5·8	1·8	2·8
8	6400	100%	1·7	1·6	2·1	3·4

*Nadir as granulocytes/cmm
†Nadir as % of original peripheral granulocyte count
‡Ratio of dis/min of ^{3}H-TdR incorporated per 30 min into acid-insoluble material per 10^6 nucleated cells to the similar measurement for ^{3}H-UdR in same cells.

10^{-5}M, 1×10^{-4}M, 5×10^{-4}M and 1×10^{-3}M. The limited amount of nucleated cells available for the experiments did not permit us to study the effects of intermediate drug concentrations.

When the degree of inhibition of colony formation of the various cell samples was matched against the nadir of peripheral granulocyte count obtained following five-day 5FU infusion as a percentage of the initial granulocyte number, a definite pattern emerged (Fig. 5). The two patients with the most marked granulocytic depression (the granulocyte count at nadir being 6% and 35% of initial granulocytic count) were the ones whose CFU-C's were most markedly inhibited by the lowest 5FU concentration of 1×10^{-5}M. On the other hand, the cells of two patients who did not show any significant depression of their granulocyte count after five days of 5FU infusion showed the least degree of colony inhibition when their cells were exposed to the highest drug concentration of 1×10^{-3}M. Furthermore, three patterns of dose-survival curves were observed, one in the two patients 7 and 8, who had no myelotoxicity, another in patients 3, 4 and 5 and a third in patient 2.

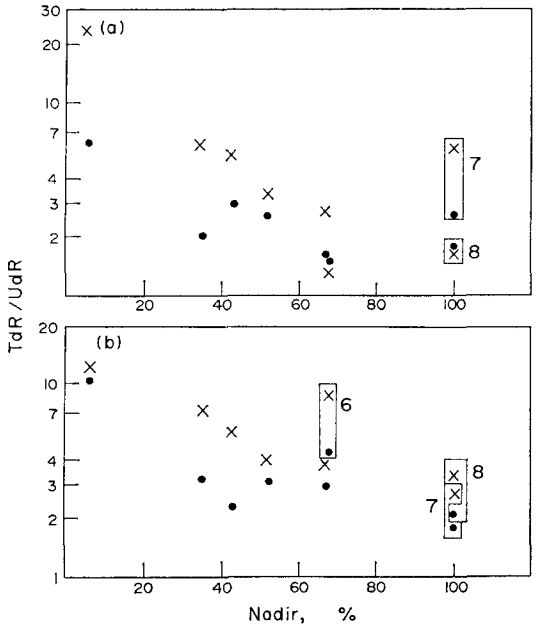

Fig. 1. Plot of ratio of the rate of ^{3}H-TdR and ^{3}H-UdR incorporation for each patient before (A) and after (B) therapy with 5FU infusion against the nadir as a % of the original granulocyte count for that patient. The numbers 6, 7 and 8 refer to those patients.

●: *no 5FU added to cells* in vitro.
×: *5FU added to cells* in vitro.

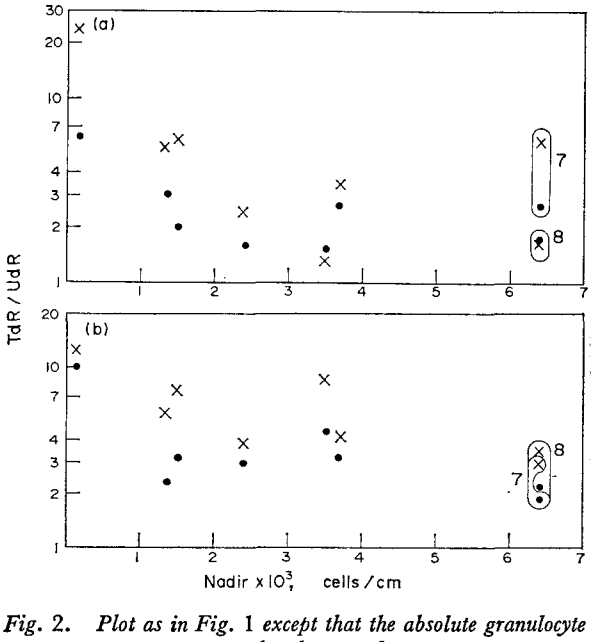

Fig. 2. Plot as in Fig. 1 except that the absolute granulocyte count has been used.

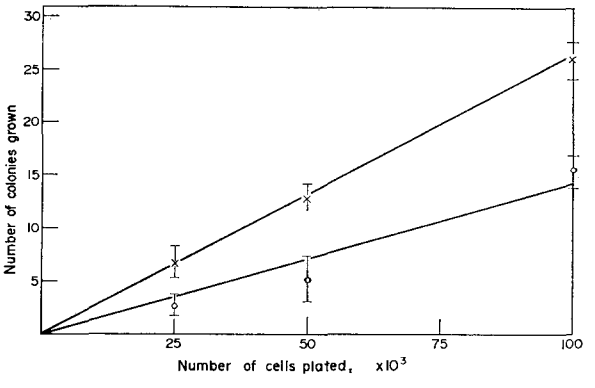

Fig. 3. Number of CFU-C's formed in relation to the number of nucleated cells plated. ×, control; ○, cells treated with 1×10^{-5}M 5FU for 1 hr. The bars represent standard error.

DISCUSSION

Effective chemotherapy of cancer requires that a maximum number of malignant cells be killed while enough normal cells survive to maintain life. This differential killing has been measured by comparing the decrease in colony forming units of untreated tumor and bone marrow cells after their injection into lethally irradiated mice [8, 9]. Also, Ogawa, Bergsagel and McCulloch, have applied the same principle to study the effect of chemotherapeutic agents on tumor and bone marrow cells in culture [10, 11]. Attempts have also been made to predict whether malignant cells are sensitive to chemotherapeutic agents by exposing the cells to the drug *in vitro*, and measuring some biochemical or biological parameter [12]. Fewer studies have attempted to predict bone marrow toxicity in patients treated with such

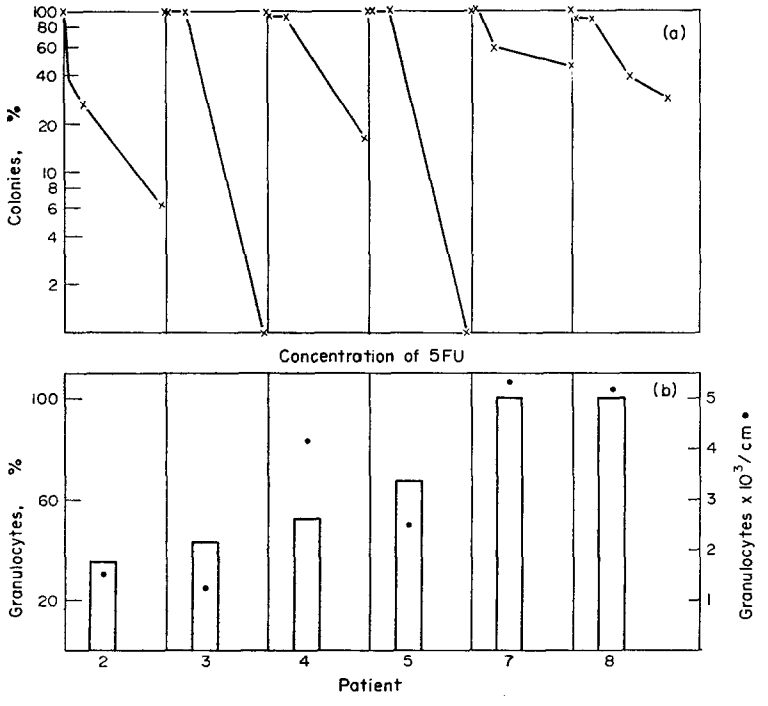

Fig. 4(a). Dose-survival curves of CFU-C's from marrow cells of six patients. The cells from patients 2, 3, 4, 5 and 7 were treated with the three concentrations of 5FU, $1 \times 10^{-5}M$, $1 \times 10^{-4}M$ and $1 \times 10^{-3}M$ for 1 hr. Cells from patient 8 were given an additional treatment with $4 \times 10^{-4}M$ 5FU. Patients 1 and 6 were studied only with one concentration, $1 \times 10^{-5}M$, and these results are not shown here.

Fig. 4(b). Peripheral granulocyte count at nadir expressed as percentuge of count before treatment (boxes) and as an absolute value, ●.

agents. In one such study, Herbert *et al.* [13] measured TdR incorporation into tumor and marrow cells in the absence and presence of drugs, including 5FU. They observed no depression of TdR incorporation with marrow cells in the presence of 5FU, as our results confirm. Herbert *et al.* [13] did not measure UdR incorporation. Absolute rates of ^{3}H-TdR and ^{3}H-UdR incorporation varied over a 20 to 14 fold respectively before treatment in the absence of 5FU. The TdR/UdR ratios varied only 4 fold, indicating that both the "salvage" and the *de novo* pathways increased together and thus did represent increased DNA synthesis in these cases.

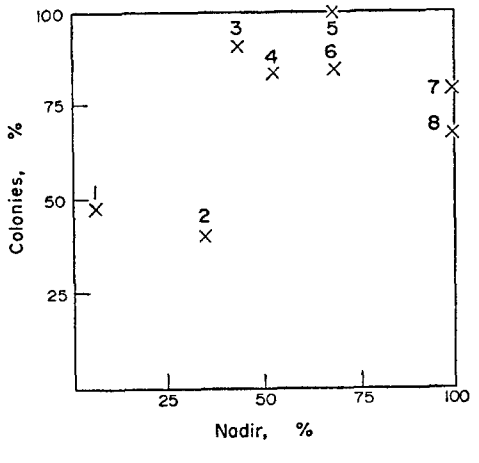

Fig. 5. Relation between marrow toxicity as shown by the nadir of the peripheral granulocyte count after treatment with 5FU as a percentage of the count before treatment and the inhibition of CFU-C formation by $1 \times 10^{-5}M$ 5FU. The numbers 1 to 8 refer to the patient studied.

After the addition of 5FU, however, a variation of 17 fold occurred in this ratio and high ratios correlated in most patients with marrow toxicity. This could be explained if such cases had a pool of thymidine triphosphate (TTP) susceptible to 5FU inhibition. This would reduce the TTP pool in these cells as shown by Tattersall and Harrap in other cells [14] and produce inhibition of growth of marrow cells. At the same time, the specific radioactivity of ^{3}H-TTP, formed from ^{3}H-TdR, would be increased due to the small pool of TTP so that the rate of ^{3}H-TdR incorporation would apparently increase. As well, ^{3}H-UdR incorporation would increase due to the inhibition of its conversion to ^{3}H-thymidylate. Thus the ratio of TdR/UdR rates would show the greatest increase in those cells most sensitive to the effect of 5FU. A significant decrease in the inhibition by 5FU of ^{3}H-UdR incorporation occurred after infusion of the drug into

patients for five days. The reason for this is not clear. The effect was present whether or not there was an increase in DNA synthesis in the same cells as shown by the rate of ^{3}H-UdR or ^{3}H-TdR incorporation in the absence of the drug *in vitro*. This suggests that the proliferative state of the marrow was not a factor in the induced resistance to 5FU or in toxic effects observed on peripheral granulocytes. Also the rate of ^{3}H-UdR incorporation varied in the same way as the ^{3}H-TdR incorporation in post-treatment samples, indicating that the induced resistance to 5FU did not result from a conversion from the *de novo* to the "salvage" pathway in those cells.

Granulocytic colony formation was another index of drug toxicity since the most marked inhibition *in vitro* was seen in those cases with most marked depression of the peripheral granulocyte counts. This probably resulted from similar *in vitro* and *in vivo* injury to granulocytic stem cells by the drug.

While the biochemical parameters we measured correlated with the observed toxicity, other parameters also might. For example, Nahas, Savlov and Hall [15] have found differences in the ratio of the activities of phosphoribosyl transferase and thymidine kinase in colon tumors and normal mucosa which related to tumor responsiveness. These and other enzyme activities in marrow cells might also correlate with toxicity of 5FU.

All of our dose-survival curves for marrow cells treated with 5FU are biphasic. This is different from the dose-survival curves obtained by Ogawa *et al.* [11] who studies the effect of 5FU in concentrations between 1×10^{-2}M and 3×10^{-2}M and obtained exponential curves. The difference between our findings and theirs might be due to the wider range of concentrations used in our experiments. Our lowest concentration, 1×10^{-5}M, is 1,000 times lower than theirs. Furthermore, their dose-survival curve was plotted only for concentrations between $1{\cdot}0 \times 10^{-2}$M and $3{\cdot}0 \times 10^{-2}$M while we used a much wider range of drug concentration, $1{\cdot}0 \times 10^{-5}$M to $1{\cdot}0 \times 10^{-3}$M. Because our dose-survival curves were plotted over that range of 5FU which spans the range of 5FU concentrations obtained during clinical use of 5FU [16], our results more correctly reflect the response of human CFU-C to varying concentrations of 5FU. Thus, the dose-survival curve of human CFU-C is biphasic when plotted over a wide concentration of 5FU but is exponential over a narrower range of concentration.

In addition to the biphasic nature of the dose–survival curves, the pattern of the curves varied suggesting a heterogenous lethal action of 5FU. Cells grown in the presence of TdR and/or uridine (UR) showed a change in the shape of their dose–survival curves which become less steep as well [5]. The difference in the patterns of the dose-survival curves that we have observed cannot be explained by a varying content of nucleosides and deoxynucleosides in the medium used, since these had been added in excess of those found, for example, in fetal calf serum [5]. A possible explanation therefore is that the cells from some patients could be more easily rescued by these agents than the cells from others. The dose–survival curves of patients 7 and 8, for example, suggests that these cells were more protected against the lethal effects of 5FU. To support this thesis, similar experiments could be done, using cells treated with 5FU, in medium with and without added nucleosides and deoxynucleosides. In our case, all cell samples were plated in methyl-cellulose enriched with nucleosides and deoxynucleosides, hence the colony forming cells of patients showing toxic effects perhaps were unable to be rescued both *in vivo* and *in vitro* from the lethal effects of 5FU. Conversely, colony forming cells of patients who demonstrated some degree of resistance both *in vivo* and *in vitro* to the effects of 5FU may have been rescued with TdR and/or UR.

Although a possible correlation exists between the sensitivity of granulocyte colony forming ability to 5FU and in clinical marrow toxicity to the drug, this measurement takes too long to be useful during infusion unless the marrow cells were tested and the result obtained before the infusion began. The biochemical parameters described, however, can be obtained more rapidly. If marrow toxicity could be quantitated in these ways and in terms of 5FU concentration in the patients' blood and related to the dose of 5FU to be given to patients, those with less sensitive marrows could be given higher doses of drug, and those with more sensitive marrows, lower doses. In this way more effective therapy with 5FU may be achieved.

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News and Communications

Assessment of Response to Therapy in Advanced Breast Cancer

(A Project of the Programme on Clinical Oncology of the International Union against Cancer, Geneva, Switzerland)

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The following protocol of evaluation of results is a modified version of protocol first used by the National Cancer Institute (N.C.I.) Breast Cooperative Group and by the European Organization for Research on Treatment of Cancer (E.O.R.T.C.) Breast Cooperative Group. This has now been tested extensively and is proposed to a still wider audience. It is being published simultaneously in several cancer journals.

INTRODUCTION

In 1975, a project was initiated by the Programme on Clinical Oncology of the UICC to formulate a system for the evaluation of response to treatment of advanced cancer. It was considered that a single uniform system, used internationally, would be invaluable for the accurate comparison of published results from different centres. Advanced breast cancer was selected as the first tumour for consideration because a number of different evaluation methods were already in use [1–6], and it was felt that agreement on a common system might readily be achieved. A preliminary document describing such a system was prepared which was then circulated to many workers in the field for comment, after which it was revised accordingly. The final document is now presented below. It is hoped that this system will be widely adopted and that it may eventually be used as a model for the assessment of therapy of other tumours.

1. *Aim*

These guidelines are intended for use in designing clinical trials to assess the objective response of locally advanced or metastatic mammary cancer to treatment; subjective response to treatment is not considered.

2. *Criteria of eligibility*

2.1. Histological evidence of breast cancer available for review.

2.2. Objective evidence of progression of disease, i.e. new lesions appear or existing lesions become larger.

2.3. Bulk of clinical disease must be evaluable by either direct measurement or photography and/or radiography.

3. *Exclusions*

3.1. It is recognised that many factors may affect a patient's ability to tolerate specific therapy. The following should, therefore, be considered in the selection of patients for study:

3.1.1. Debility.

3.1.2. Associated medical conditions.

3.1.3. Previous antitumour therapy (especially chemotherapy and radiotherapy).

3.1.4. Other previous or concurrent treatment.

3.2. The following are factors which may compromise seriously the evaluation of results and lead to the exclusion of patients from a study of systematic therapy:

3.2.1. Previous or current malignancies at other sites, with the exception of cone-biopsied *in situ* carcinoma of the cervix uteri and adequately-treated basal or squamous cell carcinoma of the skin.

3.2.2. Patients in whom one of the following is the sole manifestation of disease: lymphoedema, hilar enlargement, pleural effusion, ascites, metastases in the central nervous system, marrow suppression and osteoblastic skeletal lesions.

3.2.3. Previous systemic antitumour treatment with the agent(s) under study.

3.2.4. In order to avoid confusion with a withdrawal response or a delayed response to treatment it is suggested that patients within 4 weeks of endocrine ablation or of cessation of additive hormone therapy be excluded. In the case of long acting hormones or depot preparations, this period may have to be increased. When previous chemotherapy or radiotherapy has been used, the toxic manifestations of these treatments, which affect subsequent specific therapy, must have resolved. After 4 weeks the patient can be included only if there is evidence of progressive disease.

4. *Base-line studies*

4.1. *History.* A standard history should be obtained in all cases, with special attention to:

4.1.1. Stage of primary disease at the time of initial presentation, expressed in the TNM Classification [7] if possible, and its histology and pathological extent.

4.1.2. Recording of race, and dates of birth, diagnosis of breast cancer and last menstrual period.

4.1.3. The extent and date of treatment of the primary tumour and the time of first recurrence should be noted. The date of first recurrence should be given as the time of documentation of the first sign, not symptom, confirmed subsequently to be a recurrence.

4.1.4. Dates, agents and the recorded result of previous treatment of recurrent breast cancer.

4.1.5. Concurrent diseases.

4.1.6. Other past and present treatment.

4.2. *Performance.* An estimation of performance may be an important part of evaluation. The Karnofsky system [8] would be appropriate or the following five-grade system could be used:

Grade	Performance
0	Fully active, able to carry on all usual activities without restriction and without the aid of analgesia.
1	Restricted in strenuous activity but ambulatory and able to carry out light work or pursue a sedentary occupation. This group also contains patients who are fully active, as in Grade 0, but only with the aid of analgesics.
2	Ambulatory and capable of all self-care but unable to work. Up and about more than 50% of waking hours.
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	Completely disabled, unable to carry out any self-care and confined totally to bed or chair.

4.3. *Physical examination.* Before starting new treatment, a full physical examination including measurement of height and weight should be done on all patients. Special attention should be directed to tissues or organs where toxic manifestations of treatment might be expected to develop.

Superficial and palpable lesions should be measured directly (in centimeters) along two axes—one being the longest and, the other, the longest perpendicular to it. Callipers may improve the accuracy of the measurements.

4.4. *Photographs.* Colour photographs of all visible lesions together with identifications, date and rule (centimeters) should be taken.

4.5. *Radiographs.* The following sites should be radiographed for baseline information with standardised exposures if possible: chest, skull, total spine, pelvis, femora, and an isotopic bone scan should be done.

4.6. *Laboratory studies.* Because therapeutic agents and/or cancer may affect normal function, appropriate investigations should be carried out to evaluate cardiac, pulmonary, metabolic, hepatic, renal, endocrine and marrow status before treatment is started.

4.7. *Additional studies.* When appropriate and feasible the following might be helpful: radio-isotope scans of brain, bone and liver, computerised axial transverse (C.A.T.) scans, mammography, bone marrow biopsy, laparoscopy, thoracoscopy, hormone receptor assays, potential markers of disease, e.g. CEA HCG, dimethyl-guanosine, ultra-sonography, im-

munological evaluation, hydroxyproline excretion.

5. *Measurement of lesions*

Normally this will be by physical examination, photography or radiography.

5.1. *Breast.* Measurements should be made as in 4.3 and superficial lesions should be photographed. Measurements from mammography may be useful. Diffuse infiltration of the breast is difficult to assess. It is recommended that cutaneous marks be made 10 cm apart, centered on the nipple. The distance between these marks is measured after digital compression of the breast and recorded as "compression at 10 cm = x cm".

5.2. *Skin.* Individual lesions should be measured as in 4.3. Diffuse lesions should be recorded by photography.

5.3. *Lymph nodes.* (a) superficial nodes: if possible, measurement as in 4.3, otherwise in one dimension. Xeroradiography may be useful.

(b) mediastinal nodes: chest X-ray, tomograms.

Generally, comparison of mediastinal size during treatment may be possible by comparing radiographs, and the width of the mediastinum could be taken as a unidimensional measurement at a stated level. The site of mediastinal nodes should be confirmed by a lateral chest X-ray.

(c) lymphoedema: measurement of limb circumference at a stated level above and below the olecranon.

5.4. *Bone.* An assessment of skeletal involvement should be made and individual lesions can be evaluated from selected films. Comparison of sequential bone scans may provide additional information. It should be noted that other processes can produce increased uptake of radionucleides in bone scans and that both progression and regression of lesions may be reflected by an increase in uptake.

5.5. *Lung.* (a) nodular: measurement in two dimensions from radiograph whenever possible.

(b) diffuse: this should be evaluated and compared from serial chest X-rays. It is recognised that comparisons may sometimes be difficult.

5.6. *Pleural effusion.* Pleural effusion should be recorded by chest X-rays (PA and lateral). Frequency and volume of thoracenteses should be noted.

5.7. *Liver.* Histological confirmation of hepatic metastases is desirable when this is the only site of recurrence.

Clinical measurement of the liver should be done with the patient in the supine position. The inferior border of the liver should be measured as a vertical distance below the costal margin or xiphisternal notch, at a fixed distance from the mid-line and/or at the mid-line. The same reference point must be used at all examinations in the same patient. The phase of respiration should also be the same. This will normally be either quiet respiration or deep inspiration. It should be remembered that the presence of a right pleural effusion and its subsequent aspiration can affect apparent liver size. Sequential liver scans can be used for comparison.

5.8. *Ascites.* The girth of the abdomen should be measured at a fixed point. Weight should be recorded periodically. Frequency and volume of required abdominal paracenteses and the use of diuretics should be noted. Increased adiposity may make serial measurements invalid. Ultrasonography and CAT scans may provide additional information.

5.9 *Abdominal mass.* Measurement in two dimensions with callipers where possible. If feasible, ultra-sound and computerized axial transverse scans should be done.

5.10 *Nervous system.* Neurological deficit will be recorded in the physical examination but should not be used to quantify response. Sequential CAT scans may be useful.

6. *Recording of lesions*

This should be done by anatomical site. Dimensions should be stated when applicable and the method of evaluation stated (direct measurement, photograph, radiograph, other).

It is recognised that pleural effusion, ascites, hepatomegaly, pulmonary shadows, etc., may be the result of non-malignant processes. When possible, histological proof of involvement should be obtained if these abnormalities are to be used for evaluation of response or stratification.

6.1. *Soft tissue.* Breast—ipsilateral; contralateral.

Skin—intracutaneous; subcutaneous.

Lymphoedema.

Lymphatic location stated; mediastinal and intra-abdominal nodes excluded here (see 6.3).

6.2. *Bone.* Sites recorded (state whether lytic and/or blastic).

6.3. *Visceral.* Lung (nodular or diffuse), pleura (nodules and/or malignant effusion), mediastinal and intra-abdominal nodes, liver, ascites, abdominal or pelvic masses, central nervous system.

7. *Stratification*

Controlled trials are important in clinical research. Although randomization eliminates bias and selection on the part of the investigator, considerable disparity between groups under study can still arise. Even when no disparity arises, the comparison of treatments can be made more precise by taking prognostic variables into account in the statistical analysis. It is recommended that patients should be classified according to certain factors known to influence prognosis or response to therapy. These factors can be used to stratify patients, using a system of allocation which balances numbers allotted to different treatments within each stratum; alternatively, with non-stratified allocation, they can be used as a basis for statistical analysis of the results of the trial. The factors chosen will depend on the treatment under study. The following may be considered.

7.1. *Menopausal status.* There are three physiological categories of menopausal status which may be classified in various ways. The following is suggested:

7.1.1. Pre-menopausal—a menstrual period has occurred within the previous year.

7.1.2. Early post-menopausal—last period: 1–5 yr.

7.1.3. Late post-menopausal—last period $>$ 5 yr ago.

Women who have had a hysterectomy with one or both ovaries left in place may be excluded or considered pre-menopausal if $<$ 50 yr of age and post-menopausal if $>$ 55 yr of age (7.1.3.); those aged 50–55 yr are classified as 7.1.2. Vaginal cytology and/or hormone studies may clarify the true menopausal status. Young women who have had an artificial menopause should be excluded or considered separately.

7.2. *Disease-free interval.* (i.e. time from treatment of primary tumour by surgery or radiotherapy to time of first recurrence, see 4.1.2.).

7.2.1. No free interval.
7.2.2. $<$ 2 yr.
7.2.3. $\geqslant$ 2 yr.

All patients presenting with Stage IV(M_1) disease have no free interval. Patients receiving radiotherapy as the sole treatment of the primary tumour should be stratified separately.

7.3. *Other groups.* When appropriate, other factors can be chosen for stratification, e.g. site of disease, age, performance, histological grade, previous therapy, residual toxicity, hormone receptors, immune status etc.

8. *Follow-up studies*

8.1. The standard follow-up time for patients under study will normally be 4 weeks, but it is recognised that, for specific protocols (e.g., chemotherapy), this may have to be modified. However, it is important that each group in a controlled study be evaluated regularly at similar intervals.

8.2. Base-line studies should be repeated regularly at intervals of not more than 6 months, unless symptoms develop which demand earlier examination.

8.3. Photographs should be repeated every 3 months or sooner if changes occur.

9. *Definition of response*

9.1. *Measurable lesions.* Ideally, all lesions should be measured at each assessment. When multiple lesions are present, this may not be possible and, under such circumstances, a representative number of eight or more lesions may be selected for measurement.

9.1.1. In the case of *bidimensional lesions,* regression will be defined as when either: (i) all lesions disappear, (ii) the sum of the products of the diameters of each individual lesion, or those selected for study, decreases by 50% or more, with no lesion increasing in size.

In each case, no new lesions should appear.

Progression is defined as when either: (i) new lesions appear, (ii) there is a 25% or more increase in the sum of the products of the diameters of each lesion measured, except that if an increase of less than 25% makes additional treatment necessary, this is also regarded as progression.

9.1.2. *For unidimensional lesions,* in the case of regression the same rules apply as in 9.1.1., except that regression is taken as a decrease of 50% or more in one measurement. In situations such as infiltration of the breast, liver involvement and mediastinal enlargement, objective regression is a 50% or greater decrease in that measurement which is regarded as being in excess of that usual for the site under consideration.

9.2. *Evaluable, but non-measurable lesions.* (e.g. osseous metastases, pulmonary infiltration, pleural effusion, skin infiltration). Serial evidence of appreciable change documented by radiography or photography must be obtained and be available for subsequent review. The assessment must always be objective.

Pathological fractures or collapse of bones are not necessarily evidence of progressive disease.

Neither the development nor healing of skin ulcers should be taken as sole evidence of change.

10. *Categories of response*

10.1 *Objective regression.*

10.1.1. *Complete response*—disappearance of *all* known disease. In the case of lytic bone metastases these must be shown radiologically to have calcified.

10.1.2. *Partial response*—$\geqslant$ 50% decrease in measurable lesions as defined in 9.1.1. and 9.1.2., and objective improvement in evaluable, but non-measurable lesions. No new lesions. It is not necessary for every lesion to have regressed to qualify for partial response, but no lesion should have progressed.

10.2. *No change.* Lesions unchanged (i.e. $<$ 50% decrease or $<$ 25% increase in the size of measurable lesions).

Note: If non-measurable, but evaluable lesions represent the bulk of disease and these clearly do not respond, even though measurable lesions have improved, then this is considered as "no change" not "objective regression".

10.3. *Progressive disease.*

10.3.1. *Mixed*—some lesions regress while others progress or new lesions appear.

10.3.2. *Failure*—progression of some or all lesions and/or appearance of new lesions. No lesions regress.

11. *Duration of response*

In a patient who has an objective regression, this is to be dated from the start of therapy until either new lesions appear or any one existing lesion increases by 25% or more above its smallest size recorded.

It is essential to categorize a patient as having a regression at a stated time. It is also essential that *all* baseline studies should have been repeated at this time.

12. *Survival*

Survival dated from time of commencement of treatment to death should be recorded.

13. *Extramural review*

It is recommended that the records of all patients under study be assessed by extramural reviewers.

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Europ. J. Cancer Vol. 13, p. 95. Pergamon Press 1977. Printed in Great Britain

Announcements

ONE-WEEK CANCER CHEMOTHERAPY COURSE SLATED FOR DECEMBER

Memorial Sloan–Kettering Cancer Center will present a one week course in Cancer Chemotherapy, 3–10 December, 1976.

This course is designed principally for physicians interested in medical oncology and clinical investigations in cancer chemotherapy. It will include lectures and panels on screening methods, pharmacological techniques and methods for clinical evaluation of potential chemotherapeutic agents. The chemistry and pharmacology of agents used in the treatment of cancer will be reviewed. There will be a strong emphasis on disease oriented "workshops" which will consider optimal methods of patient management relating medical oncology to other relevant disciplines.

Applications should be submitted as soon as possible as enrollment is limited. The fee of $150 is payable at the time of application. Requests for registration forms and program details should be addressed to CHARLES W. YOUNG, M.D., Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, N.Y. 10021, U.S.A.

SYMPOSIUM—"PLATELETS: A MULTIDISCIPLINARY APPROACH"

An International Symposium on "Platelets: a multidisciplinary approach" will be held in Florence (Italy), on 28–30 September, 1977.

The scope of this Symposium is to focus attention on platelets both as a model in pharmacology, biochemistry, immunology, inflammation and related fields and as a cell involved in physiopathological phenomena not directly connected to haemostasis and thrombosis mechanisms. Internationally acknowledged experts have been invited to present and discuss data, hypotheses, problems.

The following Sessions are scheduled:

1. Platelet morphology, biochemistry, pharmacology: introductory lectures.
2. Platelet as a model of smooth muscle cell.
3. Platelets and endothelium.
4. Platelet as an inflammatory cell.
5. Platelet as a model of monoaminergic nerve endings.
6. Platelets and immunological reactions.
7. Platelets, cancer growth and metastasis formation.

Scientific Advisory Committee: G. de Gaetano, S. Garattini, P. M. Mannucci, M. Verstraete.

Organizing Secretariat: Fondazione Internazionale Menarini, Piazza del Carmine, 4-20121 Milano, Italy—Tel. (02) 87.49.32.

For further information, please contact:
Dr. G. de Gaetano, Laboratory for Haemostasis and Thrombosis Research; Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62—20157 Milano (Italy); Tel. (02) 35.54. 546.

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Recent Journal Contents (1977)

International Journal of Cancer *January, 1977*

Human Cancer

I. N. Chernozemsky, I. J. Stojanov, T. K. Petkova-Bocharova, I. G. Nicolov, I. V. Draganov, I. I. Stojchev, J. Tanchev, D. Naidenov and N. D. Kalcheva: Geographic correlation between the occurrence of endemic nephropathy and urinary tract tumours in Vratza district, Bulgaria.

E. A. Samayoa, F. C. McDuffie, A. M. Nelson, V. L. W. Go, H. S. Luthra and H. W. Brumfield: Immunoglobulin complexes in sera of patients with malignancy.

S. H. Golub, A. B. Forsythe and D. L. Morton: Sequential examination of lymphocyte proliferative capacity in patients with malignant melanoma receiving BCG immunotherapy.

H. Ben-Bassat, N. Goldblum, S. Mitrani, T. Goldblum, J. M. Yoffey, M. M. Cohen, Z. Bentwich, B. Ramot, E. Klein and G. Klein: Establishment in continuous culture of a new type of lymphocyte from a "Burkitt-like" malignant lymphoma (line D.G.-75).

B. M. Vose and M. Moore: Reactivity of peripheral blood leucocytes against human foetal cells. II. Cytotoxic potential of preparations enriched or depleted of different leucocyte populations.

J. C. Rutherford, B. A. J. Walters, G. Cavaye and W. J. Halliday: A modified leucocyte adherence inhibition test in the laboratory investigation of gastrointestinal cancer.

J. Hesse, P. H. Levine, P. Ebbeson, R. R. Connelly and C. H. Mordhorst: A case control study on immunity to two Epstein–Barr virus-associated antigens, and to herpes simplex virus and adenovirus in a population-based group of patients with Hodgkin's disease in Denmark 1971–1973.

K. Nooter, P. Bentvelzen, C. Zurcher and J. Rhim: Detection of human C-type "helper" viruses in human leukemic bone marrow with murine sarcoma virus-transformed human and rat nonproducer cells.

G. Klein, P. Terasaki, R. Billing, R. Honig, M. Jondal, A. Rosén, J. Zeuthen and G. Clements: Somatic cell hybrids between human lymphoma lines. III. Surface markers.

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D. Schmähl, R. Port and J. Wahrendorf: A dose–response study on urethane carcinogenesis in rats and mice.

N. Auersperg, J. B. Hudson, E. G. Goddard and V. Klement: Transformation of cultured rat adrenocortical cells by Kirsten murine sarcoma virus (Ki-MSV).

M. Essex, S. M. Cotter, A. H. Sliski, W. D. Hardy, Jr., J. R. Stephenson, S. A. Aaronson and O. Jarrett: Horizontal transmission of feline leukemia virus under natural conditions in a feline leukemia cluster household.

B. C. Veit, J. M. Jones, G. A. Miller and J. D. Feldman: Genetic association of the humoral and cellular immune responses of rats to Moloney sarcomas.

G. Giraldo, E. Beth, U. Hämmerling, G. Tarro, F. M. Kourilsky and L. J. Old: Detection of early antigens in nuclei of cells infected by cytomegalovirus, Herpes simplex virus type 1 and 2 by anticomplement immunofluorescence and a blocking assay to demonstrate their specificity.

A. S. K. Murthy, J. R. Baker, E. R. Smith and G. G. Wade: Development of hemangiosarcomas in mice fed 2-methyl-1-nitroanthraquinone.

E. Huberman and L. Sachs: DNA binding and its relationship to carcinogenesis by different polycyclic hydrocarbons.

A. A. Yunis, G. K. Arimura and D. J. Russin: Human pancreatic carcinoma (MIA PaCa-2) in continuous culture. Sensitivity to asparaginase.

British Journal of Cancer *January, 1977*

Europ. J. Cancer Vol. 13, p. 98. Pergamon Press 1977. Printed in Great Britain

Papers to be Published

SESHADRI RAJESWARI, S. N. GHOSH, P. N. SHAH and V. J. BORAH
Barr body frequency in the human breast cancer tissue. A prospective study on its prognostic value and its correlation with specific oestradiol receptors.

G. A. YOUNG and F. M. PARSONS
The effects of dietary deficiencies of magnesium and potassium on the growth and chemistry of transplanted tumours and host tissues in the rat.

J. W. PROCTOR, B. G. AUCLAIR, L. STOKOWSKI, P. W. A. MANSELL and H. SHIBATA
Comparison of effect of BCG, glucan and levamisole on B16 melanoma metastases.

A. M. VENUAT, B. DUTRILLAUX and C. ROSENFELD
A late clonal evolution of a human leukemic line: Sequential cytogenetic studies.

MARK A. WAINBERG and RICHARD G. MARGOLESE
Desensitization of effective anti-tumour immunity in guinea pigs.

D. E. SCHUMM, M. HANAUSEK-WALASZEK, A. YANNARELL and T. E. WEBB
Changes in Nuclear RNA transport incident to carcinogenesis.

R. I. NICHOLSON, P. DAVIES and K. GRIFFITHS
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D. GANGJI, W. PILLOY, J. C. HEUSON and J. FRÜHLING
Regression of massive liver involvement by metastatic breast cancer after chemotherapy as monitored by scintigrams using a stereological method.

P. BENTVELZEN and J. BRINKHOF
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E. R. ATKINSON
Letter to the Editor: Comments on Yerushalmi's article (Reference 1).

M. F. ROUSSEAU-MERCK, M. N. LOMBARD, C. NEZELOF and H. MOULY
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NEIL M. BORTHWICK, DOUGLAS W. WILSON and PHILIP A. BELL
Carcinoembryonic antigen (CEA) in patients with breast cancer.

JUNG KOO YOUN, DANIELLE LE FRANCOIS, MAUD SANTILLANA, GILBERT HUE and GEORGES BARSKI
Effect of BCG treatment on the evolution of cell-mediated immunity in mice bearing transplantable syngeneic tumors.

D. Y. WANG, R. D. BULBROOK and J. L. HAYWARD
Plasma androstenedione levels in women with breast cancer.

ALAN S. MORRISON, C. RONALD LOWE, BRIAN MACMAHON, BOŽENA RAVNIHAR and SHU YUASA
Incidence risk factors and survival in breast cancer: Report on five years of follow-up observation.

P. D. BROWN and P. F. ZAGALSKY
Studies of the effects of busulphan on the regeneration of rat liver.

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Barr Body Frequency in the Human Breast Cancer Tissue.

A Prospective Study on its Prognostic Value and its Correlation with Specific Oestradiol Receptors

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Abstract—*This communication describes the study on the Barr body frequency from* 93 *tumour specimens. From the results, it appears that this biological determinant provides an accurate and reliable prediction about the clinical course of the disease in a given patient. Analysis of Barr body frequency* vis-à-vis *oestradiol receptors in* 50 *unselected breast cancer tissues did not reveal any significant correlation.*

INTRODUCTION

RECENT clinical data have pinpointed that breast cancer is a systemic disease and it is the early recurrence followed by metastases that decides the quality of life and duration of survival of the patient. Friedell *et al.* [1] analysed various clinical, pathological, and urinary steroid variables in patients with breast cancer treated by radical mastectomy and found that the extent of regional lymph node involvement appeared to be the most important single factor of prognostic significance for early recurrence after primary surgery. Ideally speaking, one would prefer some intrinsic characteristics of the tumour cells in a given case for prognostic value, but it is surprising to note that very little work has been published so far on this subject. A preliminary communication from this laboratory [2] on a retrospective study has highlighted the prognostic value of Barr body frequency in tumour cells in a given patient—the parameter used being 2 yr disease free interval of distant metastases.

Further, a group of investigators [3–7] have recently suggested that tumours showing high Barr body frequency are oestrogen-dependent. So a study wherein correlation between the Barr body percentage and oestrogen receptors is determined appears to be necessary for obvious reasons. The purpose of this communication therefore is two-fold; (a) to confirm preliminary observations reported earlier [2] by a prospective study and (b) to assess the correlation, if any, between the frequency of Barr body and the presence of significant amounts of specific oestradiol receptors in the tumour tissue of an individual patient.

Accepted 27 August 1976.

MATERIAL AND METHODS

In all, 93 patients were studied. When these cases were classified according to the UICC, AJC, TNM classification of Geneva, 1972, 36% belonged to Stage 1, 50% to Stage II, and the remaining 14% to Stage III. No treatment was given to any patient before operation. After radical mastectomy, only those patients exhibiting involvement of axillary nodes underwent Cobalt therapy. The histological diagnosis in 95% of these tumours was infiltrating duct carcinoma—Grade III. Although the patients' age ranged from 21 to 77 yr, the majority of them were between 35 and 55 yr old.

In order to assess the recurrence of the disease, patients were regularly followed-up once every 3 months after radical mastectomy. Following careful clinical scrutiny, in suspicious cases, lungs and bones were X-rayed. Liver scans were carried out whenever it was deemed necessary. However, bone scintigraphies were not carried out before or after the primary operative treatment due to lack of this facility at our hospital.

Smear preparations were made from 93 fresh tumours obtained for frozen section at the time of mastectomy. Smears were fixed in ether:alcohol (1:1) for 24 hr and stained with 1% cresyl echt violet. In each tumour, at least 300 nuclei were counted by two independent observers under oil immersion, and the percentage of Barr body was scored. There was no significant discrepancy in the percentage counts of Barr bodies in individual smears as counted by two independent observers. As reported earlier [2] tissues having less than 20% Barr body constituted the "negative group" and those showing above 20% were classed under the "positive group". The age distribution of the patients in the "positive" and "negative" groups was similar.

In addition, 10 unselected tumour explants were grown in organ culture (using McCoy 5A medium containing 20% foetal calf serum) for a period of 25 days and the Barr body frequency was studied before and after culture. The viability of the tumour cells was checked after termination of culture by Trypan blue exclusion test.

For the specific oestradiol receptors, 50 unselected tumours were analysed. Specific binding of ^{3}H 17B oestradiol(6,7-^{3}H oestradiol Sp. Act. 40 Ci/m mole from Radiochemical Centre, Amersham, England) in the high speed cytosols were quantified using the Agar-gel electrophoresis method of Wagner *et al.* [8]. As suggested by McGuire [9] tissues with binding of oestradiol more than 3 femtomoles/mg cytosol protein were categorised in the receptor "positive" group.

RESULTS

The pattern of Barr body in a representative group of both, "negative" and "positive" tumours, did not alter significantly after 25 days of *in vitro* culture (Table 1).

When lymph node status and duration of disease-free interval up to 2 yr were compared (Table 2), there was only a border-line significance ($P < 0{\cdot}05$). There was no significant correlation between Barr body frequency in the tumour cells and lymph node status in 93 patients (Table 3). On the other hand, as shown in Table 4, the correlation between Barr body frequency and duration of disease free interval by the end of 2 yr was highly significant ($P < 0{\cdot}001$). Table 5 demonstrates the absence of correlation between the Barr body frequency and the presence or absence of specific receptors for oestradiol in 50 unselected tumour tissues ($P > 0{\cdot}5$).

Table 1. *Percentage of Barr body in the tumour tissue before and after* 25 *days of* in vitro *culture*

S. No.	Case No.	Barr body percentage	
		Fresh tumour (%)	After culture (%)
1.	AF14181	14	15
2.	AG1319	10	8
3.	AG1915	10	11
4.	AG2074	16	16
5.	AG535	24	29
6.	AG3703	22	20
7.	AG7531	50	42
8.	AG4602	14	12
9.	AF14181	12	12
10.	AG9507	10	13

Table 2. *Correlation between lymph node involvement and duration of disease free interval in* 93 *patients with proven breast cancer*

Lymph node involvement	Duration of disease-free interval	
	⩽ 2 yr	> 2 yr
Nil (37)	16	21
Yes (56)	36	20
Total	52	41

$\chi^2 = 4{\cdot}025$; $P < 0{\cdot}05$.

Table 3. *Correlation between Barr body frequency in* 93 *breast cancerous tissues and lymph node involvement in the patients*

Lymph node involvement	Groups based on Barr body frequency	
	Negative	Positive
Nil (37)	26	11
Yes (56)	41	15
Total	67	26

$\chi^2 = 0{\cdot}1094$; Not significant.

Table 4. *Correlation between Barr body frequency and duration of disease-free interval in* 93 *patients with breast cancer*

Duration of disease-free interval	Groups based on Barr body frequency	
	Negative	Positive
⩽ 2 yr (52)	47	5
> 2 yr (41)	20	21
Total	67	26

$\chi^2 = 19{\cdot}538$; $P < 0{\cdot}001$.

DISCUSSION

In our preliminary report [2] it was shown that the percentage of Barr body in different parts of a cancerous tissue was consistent as assayed by the smear method. However, report of a case of colon carcinoma [10] wherein the "negative" group of Barr body frequency was changed into a "positive" group after *in vitro* culture, and the fact that Gropp *et al.* [11] are quoted to have reported on "regaining of the sex chromatin body after culture", prompted the present investigators to test whether such a phenomenon occurs in the human breast cancer tissue. As aforesaid, even after 25 days of *in vitro* culture, there was

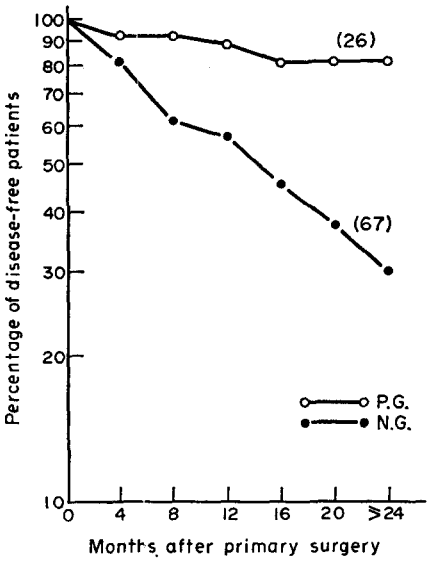

Fig. 1. *Shows the percentage of patients (irrespective of their lymph node status) with recurrence in time up to* 24 *months in the Positive Group (P.G.) and Negative Group (N.G.)*

no significant change in the Barr body frequency of the tumour tissue as compared to that seen in the fresh smears studied from 10 breast cancerous tissues belonging to both "negative" and "positive" Groups (Table 1). In other words, the fixation of the Barr body in tumours of the breast is an intrinsic, unalterable characteristic of the tumour.

After having established the consistency of the Barr body pattern in the malignant breast tissue, its significance in prognostication was considered. Our earlier publication [2], which mostly dealt with observations on a retrospective study, had established a significant positive correlation between the Barr body percentage and a 2 yr disease-free interval of distant metastases and 5 yr survival. Figure 1 graphically represents the results on this prospective study on 93 patients wherein a distinct dichotomy can be observed between patients in the "negative" and "positive" groups when followed up for 2 yr of disease-free interval. In other words, distant metastases occur in 66% of the patients in the "negative" group within 2 yr as compared to only 20% in the "positive" group and this difference is statistically significant ($P < 0{\cdot}001$). This observation was further analysed on the basis of the lymph node status in association with "negative" and "positive" tumours (Fig. 2). There was no significant difference in the percentage of patients having recurrence

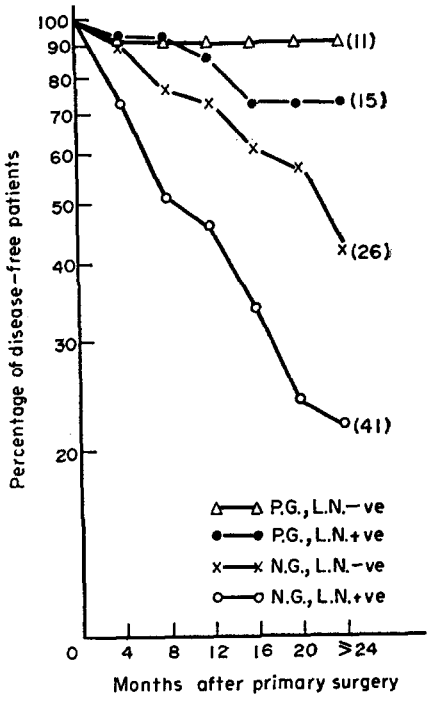

Fig. 2. *Reveals the percentage of patients with and without lymph node involvement (L.N.+and L.N.−ve) when considered along with recurrence in time up to* 24 *months in the Positive Group (P.G.) and Negative Group (N.G.)*

within 2 yr in the same group with or without lymph node involvement. This provides further evidence to indicate that Barr body frequency appears to be a more accurate and reliable parameter and convinces its superiority as a single prognostic biological determinant.

Although there is hardly any published report on the Barr body frequency in tumour cells with presence or absence of oestradiol receptors, there are a few reports [3–7] in the literature on the correlation between Barr body frequency and response to endocrine treatments in breast cancer patients. According to these investigators, patients with tumours having high Barr body frequency respond favourably to hormonal manipulations. With the current

Table 5. *Correlation between Barr body frequency and specific receptors for* 17*B oestradiol in* 50 *tumour tissues*

Receptors for 17B-oestradiol	Groups based on Barr body frequency	
	Negative	Positive
Negative (32)	19	13
*Positive (18)	10	8
Total	29	21

*Positive = 3 fmole/mg Cytosol Protein.

χ^2 = 0·0689; Not significant.

consensus [9] regarding poor response to such manipulations in patients having tumours showing absence or negligible oestrogen receptors, it becomes imperative to see if there exists any correlation between these two intrinsic characteristics of the tumour tissue in a given patient. As is evident from Table 5, at least in the 50 unselected tumours studied at this laboratory, no correlation was found to exist. In other words, it seems that Barr body frequency and presence of significant amounts of oestradiol receptors in human breast cancerous tissue are unrelated characteristics.

Further, for total management in a given patient it still remains to be established whether information on Barr body frequency and the lymph node involvement could be exploited to delineate cases for prophylactic combination chemotherapy with a hope to give a longer and better qualitative life.

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The Effects of Dietary Deficiencies of Magnesium and Potassium on the Growth and Chemistry of Transplanted Tumours and Host Tissues in the Rat

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Abstract—1. *Weanling rats on control diet or a diet deficient in magnesium and/or potassium for* 1–3 *weeks were transplanted with Yoshida or Walker tumours. Restricted intakes were given to prevent nutritional differences between the groups. Cation depletion was assessed from plasma and muscle concentrations.*

2. *Organ weights and tissue biochemistry showed that metabolic differences between control and cation deficient rats were due predominantly to cation deficiency and the tumour growth rather than other nutritional deficiencies.*

3. *Reduction in tumour size occurred in Mg deficiency (up to* 40%), *K deficiency* (30–60%) *and in combined deficiency* (45–85%). *Tumour size showed the best correlation with the combined concentration of plasma Mg and K or muscle-K.*

4. *Significant reduction in tumour K was observed only in marked combined deficiency.*

5. *Correlation between Mg and K was found in tumour but not in muscle.*

6. *Dietary depletion of both Mg and K cause the removal of K from tissues and may affect the replication and survival of tumour cells by inhibiting metabolic activity and causing the loss of intracellular K.*

INTRODUCTION

PREVIOUS investigations of patients have shown that combined dietary deficiency of magnesium (Mg) and potassium (K), particularly if enhanced by intermittent haemodialysis against a dialysis fluid low in these elements, is associated with tumour regression [1]. Studies with rodents have also suggested that tumour growth is adversely affected by dietary deficiencies of these elements. Mg deficiency alone was thought to cause a 95% growth reduction of Flexner Jobling carcinoma [2] and of Walker carcinosarcoma 256 [3]. However, growth reduction of Landschutz ascites tumour in mice occurred only when Mg and K or K alone were deficient in the diet [4]. Unfortunately, the relative weights of rodents, daily dietary intake or plasma concentrations of Mg and K were not recorded in any of these earlier experiments. Growth retardation of an adenocarcinoma in mice receiving an *ad lib* K free diet has been reported [5], but there were marked weight differences between control and deficient animals. There is considerable evidence (reviewed by White [6]) that nutritional and weight differences between control and underfed rodents may cause growth retardation of many tumours. Consequently, observed reduction of tumour growth in these earlier experiments might have been caused partly by the poor nutrition of experimental animals compared with controls. These factors have been incorporated into this investigation of the effects of Mg and K deficiency on the growth and chemistry of two transplantable tumours in rats.

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MATERIAL AND METHODS

Male, inbred, Wistar rats, 3–4 weeks from Chester Beatty Research Institute, London, were maintained on semi-synthetic diets based on those described by Heaton and Anderson [7]. The control rats received the diet containing adequate Mg (650 ppm) and K (4500 ppm) whilst all other rats received similar diet but deficient in Mg (15 ppm) or K (50 ppm) or

Table 1. *Mean body and tumour weights (±s.e.) with percentage reduction in tumour size for rats given diets deficient in Mg or K or both as compared with controls. P values are shown for the correlation between tumour size and the sum of the concentrations of Mg and K in plasma*

Experiment	Number of rats	Diet	Intake (g)	Tumour	Days fed prior to transplantation	Tumour growth (days)	Mean body weights prior to transplantation (g)		Mean weights at death (g)		% Reduction in tumour size	Tumour size *v* plasma Mg+K m-mole/l (*P* value)
							For each diet	For each experiment (c.v.)	Body	Tumour		
1.	12	Control	v	Yoshida	19	10	114±2		120±1	4·5±0·7		
	12	Mg deficient	v	Yoshida	19	10	105±3		113±3	3·5±0·3†	22	
	11	K deficient	v	Yoshida	19	10	98±2	105·2	103±4	1·8±0·3‡	60	< 0·001
	10	Mg+K deficient	v	Yoshida	19	10	104±3	(6·6)	107±4	1·5±0·3‡	66	
2.	6	Control	v	Yoshida	21	9	96±2		117±1	2·8±0·4		
	5	K deficient	v	Yoshida	21	9	81±3	83·7	80±4	2·0±0·4†	28	< 0·01
	5	Mg+K deficient	v	Yoshida	21	9	74±1	(13·4)	74±1	1·5±0·2‡	46	
3.	4	Control	9·0	Walker	6	9	75±3		91±2	12·1±0·6		
	5	Mg deficient	9.0	Walker	6	9	75±3		84±3	10·2±0·5†	16	
	5	K deficient	9·0	Walker	6	9	60±3	68·0	66±2	8·1±1·6†	33	< 0·01
	4	Mg+K deficient	9·0	Walker	6	9	62±2	(11·9)	66±3	6·7±1·4‡	45	
4.	5	Control	10·5	Walker	7	8	94±1		123±3	10·9±1·2		
	4	Mg deficient	10·5	Walker	7	8	91±2		111±3	6·6±0·7‡	39	
	5	K deficient	10·5	Walker	7	8	85±3	90·0	97±5	5·9±1·1‡	46	< 0·001
	4	Mg+K deficient	10·5	Walker	7	8	91±3	(3·8)	102±5	4·8±0·5‡	56	
5.	5	Control	v	Walker	9	8	77±2		84±2	8·2±0·8		
	5	Mg deficient	v	Walker	9	8	79±1		84±1	8·0±0·6 N.S.	2	
	5	K deficient	v	Walker	9	8	70±2	74·5	76±3	5·8±0·9‡	30	< 0·01
	3	Mg+K deficient	v	Walker	9	8	72±2	(5·6)	75±2	1·9±0·6*	76	
6.	5	Control	v	Walker	16	10	64±1		89±2	11·0±0·6		
	5	Mg+K deficient	v	Walker	16	10	65±3	64·5 (1·1)	68±2	1·6±0·3‡	85	< 0·001
7.	5	Control	v	Walker	27	9	68±1		86±2	10·2±0·9		
	5	Mg+K deficient	v	Walker	27	9	66±2	67·0 (2·1)	69±2	1·7±0·5‡	83	< 0·001

Mean tumour weights significantly lower than for control group: †$P < 0·05$; ‡$P < 0·02$; *$P < 0·001$.
v = variation of daily intake within range of 8–10 g to maintain comparable mean body weights for each group.
c.v. = coefficient of variation between the means of each group.

Table 2. Mean weights for body, tumour, kidneys, spleen, liver, lung and heart of rats maintained by pair feeding with control or Mg and K deficient diet for 28 days. Walker tumour was implanted in 30 of the rats on the 19th day

Number of rats	Diet	Tumour	Mean weight at death (g) ± Standard error							
			Body	Tumour	Tumour/body	Kidneys	Spleen	Liver	Lung	Heart
17	Control	Tumour	114† ± 1·6	10·6† ± 0·7	0·093† ± 0·06	1·21 ± 0·02	0·62† ± 0·06	5·1 ± 0·2	0·79* ± 0·03	0·80† ± 0·03
13	Mg + K deficient	Tumour	88 ± 1·9	3·8 ± 0·5	0·042 ± 0·005	2·05† ± 0·11	0·15 ± 0·02	4·5 ± 0·3	0·67 ± 0·03	0·55 ± 0·03
6	Control	No tumour	122† ± 1·5			1·16 ± 0·03	0·21 ± 0·04	5·0 ± 0·2	0·69 ± 0·04	0·57 ± 0·03
7	Mg + K deficient	No tumour	90 ± 4·3			2·27† ± 0·12	0·20 ± 0·02	4·7 ± 0·4	0·69 ± 0·05	0·51 ± 0·03

Mean weight significantly greater than for the paired group: *$P < 0·02$; †$P < 0·001$.

both. In preliminary experiments it was found undesirable to give an unrestricted amount of diet because control rats gained far more weight than deficient rats and also those receiving the Mg deficient diet were so rapidly depleted that some died within 10 days. In addition, control rats on unrestricted intakes (exceeding 15 g/day) for 3–4 weeks developed larger tumours than those underfed with only 8 g as described previously [6]. Consequently, restricted amounts of the diet that were not in excess of the appetite of any one group of rats were given, usually between 8 and 10·5 g/day. In some experiments the same daily amount of diet was given to all groups whereas in the other experiments different quantities were given to each group and this quantity was adjusted when necessary to obtain comparable mean body weights between the groups prior to transplantation.

The rats were maintained throughout on their respective diet and were given Yoshida ascites tumour (about 1×10^6 cells) subcutaneously in the inguinal region or the Walker carcinosarcoma 256 into the right flank [8] 7–27 days after commencing the semi-synthetic diet.

The animals were decapitated 8–10 days later and shed blood was collected in heparinised tubes. The tumours were dissected out and weighed. Samples of muscle and viable tumour tissue were removed for chemical analysis. Tissues were dried for 24 hr at 105°C and extracted for 24 hr with 0·15 N nitric acid for electrolyte analysis [9]. In several experiments spleen, kidneys, liver, lungs and heart were removed and weighed. Mg was determined by atomic absorption spectroscopy; Na and K by flame photometry. Urea nitrogen, calcium and inorganic phosphorus were measured on the Technicon autoanalyser, albumin by radial immunodiffusion, haemoglobin by the cyanomethaemoglobin method and chloride using a Corning-Eel chloride meter. Extracellular space of the muscle and tumour tissue was not determined. The total concentration of each element was expressed in mmol/kg of dry tissue or m-mol/l of tissue water and, therefore, does not indicate the distribution or nature of the element within the cell.

RESULTS

The effects of Mg and K deficient diets on tumour growth

Table 1 shows the percentage reduction in tumour size in rats maintained on diets deficient in Mg or K or both as compared with those receiving control diet after a total period of 2–5 weeks. The same daily intake of diet was given to all four groups in two experiments (3 and 4) both before and after transplantation with Walker tumour. In the other five experiments, different dietary intakes were given (8–10 g) to achieve comparable mean body weights between the groups prior to transplantation with Yoshida tumour (experiments 1 and 2) or Walker tumour (experiments 5–7). Comparability of weight between groups was not achieved in all experiments, but, the coefficient of variation did not exceed 13% and was less than 5·5% in experiments 4–7. However, following implantation the gain in weight of the controls was usually greater than for the rats on the deficient diets causing a slightly greater difference in the mean weights

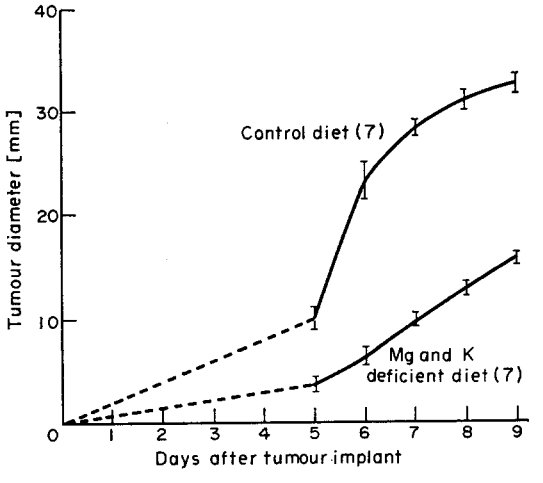

Fig. 1. Growth of Walker tumour in male rats after 19 days pretreatment. Tumour growth was markedly reduced in Mg and K deficient group. Mean values ± S.E. are shown.

at death. Table 2 compares organ weights in rats receiving either control or Mg and K deficient diet by pair feeding, both with and without tumour implants. There were no differences between the mean weight of the liver in any of the groups. However, the lung, spleen and heart were enlarged in control rats with tumours but not in those without tumours or in the Mg and K deficient group where tumour growth was retarded. The kidneys of rats receiving the Mg and K deficient diets were grossly enlarged.

Tumour size was significantly less for rats receiving the cation deficient diets than for controls. The smallest tumours occurred when both Mg and K were deficient in the diet, particularly after longer periods of depletion, (3–5 weeks) as in experiments 6 and 7, when a reduction of 85% was observed (Table 1).

Table 3. Mean concentrations of Mg, K and Na in plasma for groups of rats (Experiment 1) given control diet or a diet deficient in Mg, K or both

Plasma cations m-mole/l	Control (12)	Magnesium deficient (11)	Potassium deficient (11)	Magnesium and potassium deficient (10)
Mg	0·79	0·15‡	0·98§	0·33‡
	(0·02)	(0·01)	(0·05)	(0·04)
K	7·2	6·2†	2·2‡	2·8‡
	(0·29)	(0·18)	(0·08)	(0·12)
Na	144·0	139·0	143·0	143·0
	(2·9)	(1·5)	(1·5)	(1·8)

Mean values significantly lower than for controls:† $P < 0{\cdot}02$;‡ $P < 0{\cdot}001$.
Mean values significantly higher than for controls: §$P < 0{\cdot}001$.
Standard error given in parenthesis.

In both these experiments the mean body weights for the groups of control and deficient rats were the same at the time of transplantation and, subsequently, these rats were given the same weight of diet. The reduction of growth of Walker tumour in rats receiving Mg and K deficient diet compared with those on control diet is shown in Fig. 1 and the reduction in tumour to body weight ratio in Table 2. This effect on tumour growth occurred in all the pair feeding and weight matching experiments and was unlikely to be caused by the small nutritional differences between the groups.

Cation composition of tissues

(a) *Plasma.* Table 3 shows the mean plasma concentrations of Mg, K and Na in four groups of 10–12 rats (Table 1, experiment 1) maintained on control diet or a diet deficient in Mg, or K or both. In this experiment, 9 g/day of diet was given to each rat but after 10 days it was necessary to alter the intake of two groups to maintain comparable mean body weights for each group. The diet for the control rats was reduced to 7 g and that for the magnesium deficient rats to 8 g. Yoshida tumour was transplanted on day 20 and the rats killed on day 29.

In the other experiments similar plasma concentrations were obtained, depending on the initial weights of the rats, the period of depletion and the quantity of diet given. The lowest concentrations of Mg or K in plasma were usually in rats receiving the diet deficient in that single element, although there was a moderate reduction of each cation when both were deficient. In K deficiency, plasma-Mg was sometimes increased, Plasma-Na was similar in control and depleted rats. The biochemical data in Table 4 compares Mg and K deficient rats with controls and shows that plasma chloride was lower; urea nitrogen was increased indicating mild azotaemia; haemoglobin was not decreased probably because tumour growth was retarded; whilst calcium, inorganic phosphorous and albumin were not significantly affected.

(b) *Muscle.* Table 5 shows the mean muscle concentrations of Mg, K and Na in experiment 1 (Table 1) although similar results were obtained in the other experiments. In Mg

Table 4. Mean concentrations of Mg, K, Ca, chloride, phosphate, urea nitrogen, albumin and haemoglobin in the blood of rats maintained on control or Mg and K deficient diet for 28 days. Walker tumour was implanted on the 19th day

Number of rats	Diet	Magnesium m-mole/l	Potassium m-mole/l	Calcium m-mole/l	Chloride m-mole/l	Inorganic phosphate m-mole/l	Urea nitrogen m-mole/l	Haemoglobin % of normal	Albumin % of normal
17	Control	0·92	6·0	3·2	95	2·1	5·3	26‡	84
		(0·03)	(0·2)	(0·1)	(0·9)	(0·1)	(0·5)	(7·4)	(5·8)
13	Mg and K	0·42‡	2·5‡	3·0	78‡	1·9	10·7*	97	73
	deficient	(0·05)	(0·1)	(0·1)	(1·6)	(0·2)	(1·5)	(8·8)	(4·0)

Mean values significantly lower than for paired value: ‡$P < 0{\cdot}001$.
Mean values significantly higher than for paired value: *$P < 0{\cdot}01$.
Standard error given in parenthesis.

Table 5. Mean concentrations of Mg, K and Na in muscle for groups of rats (Experiment 1), given control diet or a diet deficient in Mg, K or both

Muscle cations m-mole/l	Control (6)	Magnesium deficient (6)	Potassium deficient (5)	Magnesium and Potassium deficient (4)
Mg	31·6	28·3†	33·5	32·4
	(1·1)	(0·7)	(1·3)	(0·7)
K	140·7	130·4†	90·8‡	84·7‡
	(1·8)	(4·2)	(3·7)	(4·0)
Na	27·7	35·3*	73·9§	64·7§
	(1·1)	(1·9)	(2·5)	(4·2)

Mean values significantly lower than for controls: †$P < 0{\cdot}05$; ‡ $P < 0{\cdot}001$.
Mean values significantly higher than for controls: *$P < 0{\cdot}01$; §$P < 0{\cdot}001$.
Standard error given in parenthesis.

deficiency alone the concentrations of Mg and K in muscle were each up to 10% less than for controls. K deficiency alone usually caused more than 30% decrease in muscle-K, but when combined with Mg deficiency the decrease was greater than 40% and in several experiments there was an increase in muscle-Mg. A decrease in muscle-Mg and/or K was always coupled by a gain in Na. The water content of muscle was about 75% and was not changed significantly by cation depletion.

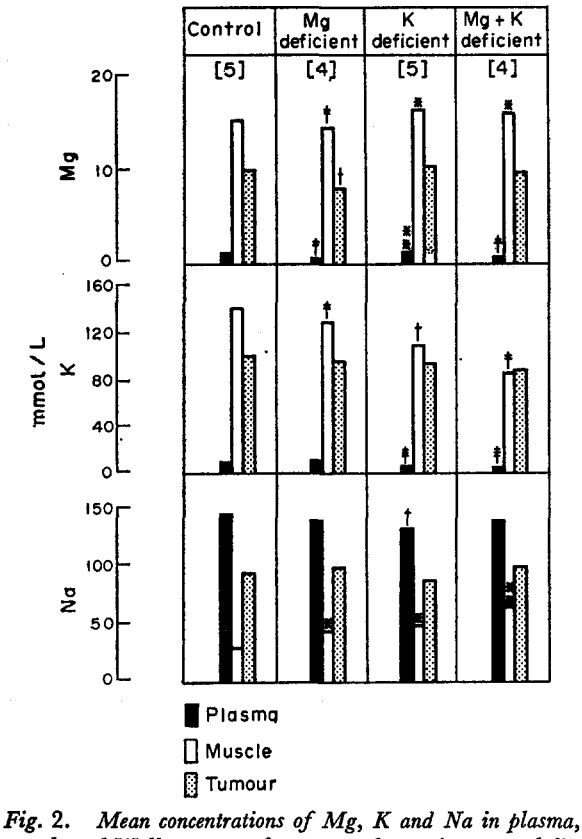

*Fig. 2. Mean concentrations of Mg, K and Na in plasma, muscle and Walker tumour for groups of rats given control diet or a diet deficient in Mg and/or K (experiment 4). Single, double or triple † or * $P < 0{\cdot}05$, 0·01 or 0·001.*

(c) *Tumour.* Figure 2 shows the mean concentrations of Mg, K and Na in tumour tissue together with the values for plasma and muscle obtained in experiment 4 (Table 1). The concentration of Mg in tumour, as in muscle and plasma, for single Mg deficiency and sometimes when combined with K deficiency, was significantly less than for values observed in rats on control diets. In the majority of experiments tumour-K, unlike the concentration in muscle and plasma, was unaffected by

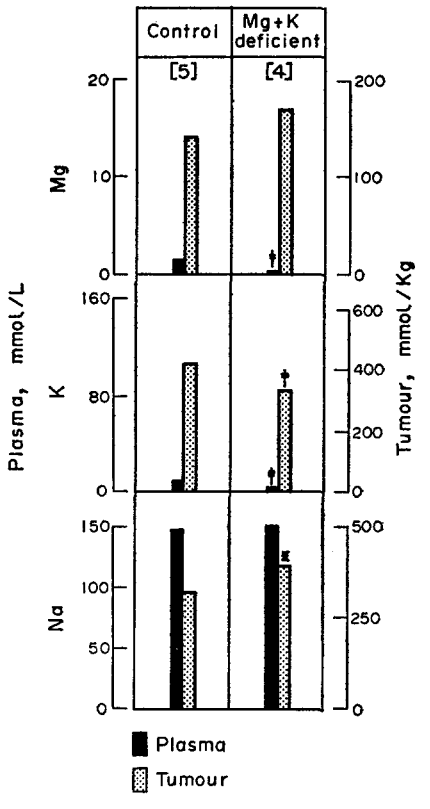

Fig. 3. Mean concentrations of Mg, K and Na in plasma and Walker tumour for groups of rats given control diet or a diet deficient in Mg and K for a longer period (experiment 6).

either single or combined deficiency. However, in experiments 6 and 7, where the sum of the plasma concentrations of Mg and K was low, with a consequent 85% reduction in tumour size, there was a significant reduction in the potassium concentration in the tumour of about 20% as compared with the 40% usually found in muscle, but also coupled by gain in Na. (Fig. 3). The water content of tumour tissue was about 80% and was not changed significantly by cation depletion.

Correlation between tumour size and Mg and/or K in plasma or muscle

There was a significant positive correlation in all experiments between tumour size and the sum of the concentrations of Mg and K in plasma (Table 1). The combined data for the experiments with the Walker tumour are shown in the correlations between tumour size and the sum of the concentrations of Mg and K in plasma or muscle (Fig. 4). These correlations include the group of rats on different diets and are not strictly homogeneous other than that they represent varying degrees of Mg and K deficiency. However, using the collective data for all the rats with a particular deficiency and transplanted with the Walker tumour there was also a correlation between tumour size and the plasma concentration of the specific cation for Mg deficient rats: tumour size vs plasma Mg; $P < 0{\cdot}001$ and Mg and K deficient rats: tumour size vs plasma Mg + K; $P < 0{\cdot}05$. There was no correlation in the K deficient rats between tumour size and plasma-K because the tumours of several rats (experiments 3 and 4, Table 1) were large despite the low plasma concentrations but the muscle-K concentrations were only 10% less than the controls indicating that the period of depletion was inadequate. However, there was a significant correlation in the K deficient rats between tumour size and muscle-K ($P < 0{\cdot}05$). Figure 5 shows the correlation for all the groups in experiments 3–5 between tumour size and muscle-K ($P < 0{\cdot}001$). Similar correlations were obtained when cations were expressed in mmol or mEq/l of tissue water or /kg of dry tissue. These results suggest that the ultimate size of a transplanted tumour in

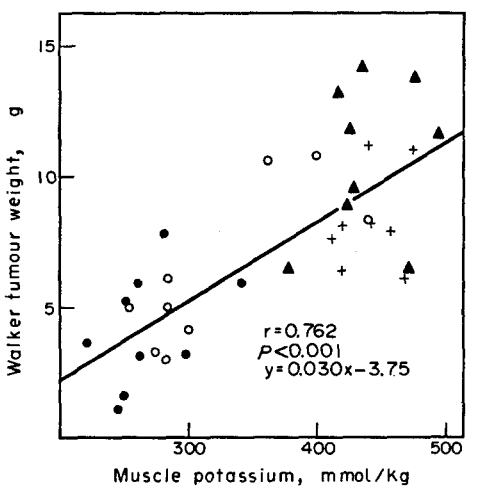

Fig. 5. Correlation between tumour size and the concentration of K in muscle of rats transplanted with Walker tumour. Diet: control ▲; Mg deficient +; K deficient ○; Mg+K deficient ●.

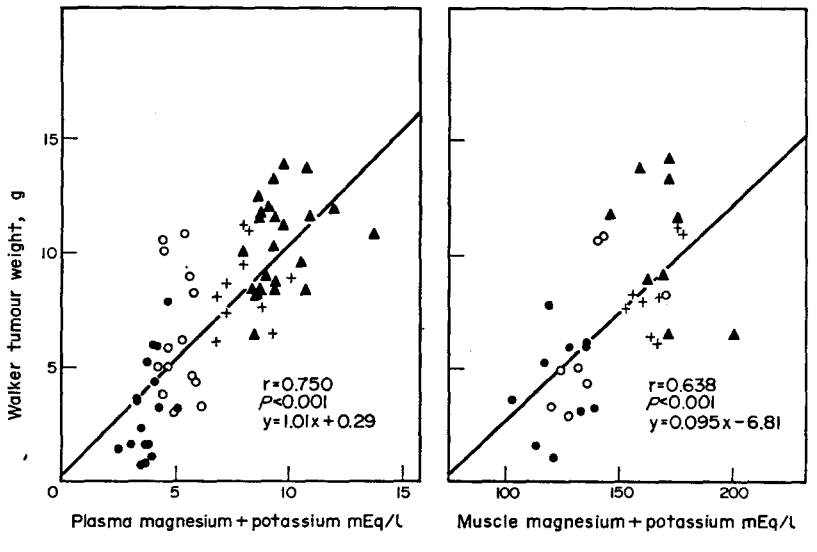

Fig. 4. Correlations between tumour size and the concentrations of Mg+K in plasma or muscle of rats transplanted with Walker tumour. Diet: control ▲; Mg deficient +; K deficient ○; Mg+K deficient ●.

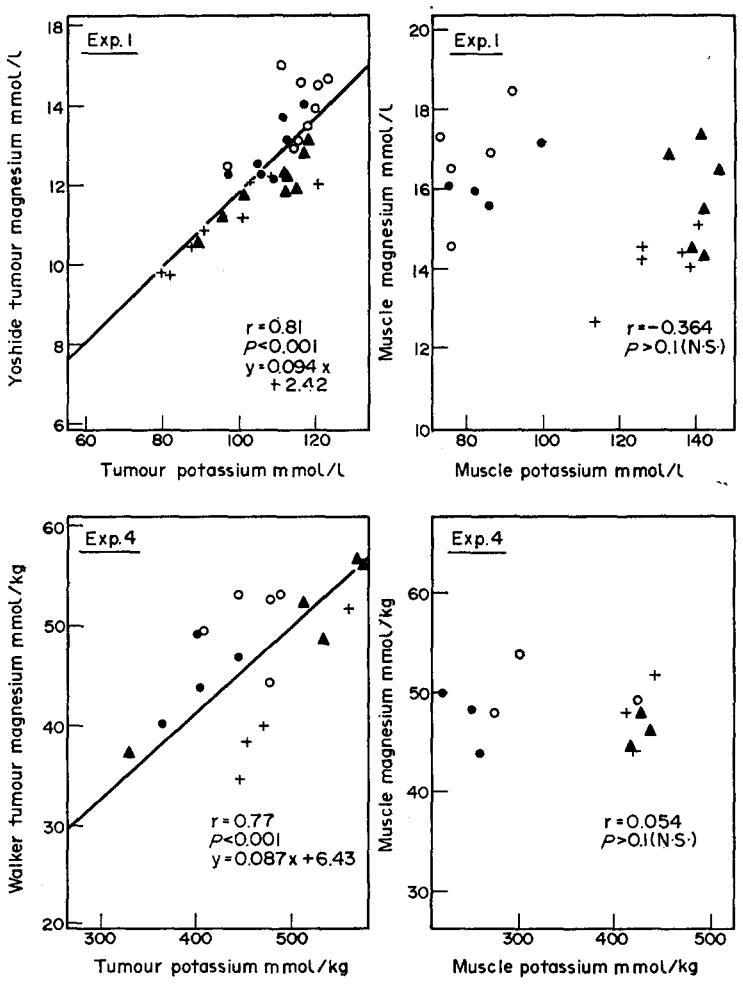

Fig. 6. Correlation between the concentration of Mg and K in both Yoshida and Walker tumour tissue but not in muscle. Diet: control ▲; Mg deficient + ; K deficient ○; Mg+K deficient ●.

the rat is partly determined by the availability of Mg and K. Maximum retardation of growth is favoured by a low concentration of Mg and K but maintained long enough to obtain maximum depletion of K in the tissues.

Differences between cation compositions of muscle and tumour tissues

In all experiments cation deficient diets caused the loss of up to 40% of muscle-K with coupled gain in Na (Mg and K deficient > K deficient > Mg deficient). Muscle-Mg was decreased by about 10% in Mg deficiency, but was frequently unaffected or even increased by the other cation deficiencies. These disproportionate losses and gains caused wide variation in the content and proportions of Mg and K in muscle. Consequently, there was no correlation between Mg and K concentrations in muscle (Fig. 6).

However, the cation deficiencies in the short term experiments (1–5) were insufficient to cause any significant loss of K from tumour tissue (never in excess of 10%). The proportions of Mg and K in tumour remained unaffected by cation deficiency and there was correlation between the concentrations of Mg and K ($P < 0{\cdot}001$) in both Yoshida and Walker tumour (Fig. 6). Similar correlations were obtained when the cations were expressed in either m-mole/l of tissue water or in mmole/kg of dry tissue and, therefore, were not caused by changes in water content.

In the combined Mg and K deficiency of longer duration (experiments 6 and 7) there was a significant loss of K from tumours (20%) frequently with gain in Na or Mg and in consequence there was no correlation between tumour Mg and K concentrations.

DISCUSSION

Mg and K deficiency both cause impairment of growth and increasing mortality between 4 and 5 weeks. Consequently, pair feeding and weight matching were necessary to minimise

nutritional differences between the different groups of rats, during the limited period of investigation. Many of the metabolic differences between the groups were characteristic of Mg and K deficiency. Modest azotaemia occurs in Mg deficiency [10]; hypochloraemia and enlargement of kidneys due to increased enzyme protein synthesis are associated with K deficiency [11]. The severe anaemia and enlargement of the spleen in the control rats with Walker tumour did not occur in those without tumours or where tumour was retarded by combined deficiency. Splenomegaly is probably an antigenic effect related to tumour size [12]. Pair feeding did not eliminate weight differences between control and combined Mg and K deficient rats particularly after tumour implantation indicating some impairment of normal tissue growth but there was no significant difference between liver weights or in plasma albumin suggesting that nutritional differences were small and unlikely to account for the reduction in tumour or tumour to body weight ratio.

Our results show that both single and combined deficiencies of Mg and K cause significant reduction in the sizes of Yoshida and Walker tumours in rats. If nutritional comparability is not maintained by pair feeding or weight matching the control and experimental animals with one another, different results may be obtained. In Mg deficiency, tumours are up to 40% smaller than matched controls but if compared with *ad lib.* fed animals the apparent growth reduction is greater and similar to that found by Sigiura [2], and Mills [3]. Potassium deficiency retards the growth of Yoshida and Walker tumours by 30–60% which is slightly less than that found in mice with an adenocarcinoma [5] and also Landschutz mouse ascites tumour [4]. However, the solid tumours, unlike the ascites tumour, are restricted most by combined deficiency (45–85%). Ryan *et al.* [4] did not give any information concerning the dietary intake or the nutritional state of their mice. Their K deficient group may have had smaller tumours than the combined deficient animals because they were more undernourished.

The retardation of tumour growth caused by Mg and/or K deficiency may be predicted from the correlation between tumour size and plasma concentrations, if the depletion is of sufficient duration (Fig. 4). Mg deficiency alone may affect growth due to the low Mg concentration in plasma, muscle and tumour, however, it also causes loss of K from muscle. Consequently, in all the cation deficiencies tumour growth is retarded by the decrease in the plasma Mg and K causing loss of K from muscle with coupled gain in Na (Mg + K deficiency > K deficiency > Mg deficiency) (Fig. 5). Combined deficiency is unlikely to be merely an additive depletion of the two elements as the Mg concentrations of tumour, muscle and plasma were sometimes increased. The disproportionate losses of K and gain in Mg of muscle caused wide variation in the content and proportions of Mg and K, consequently, there is no correlation between muscle-Mg and K (Fig. 6).

In contrast, tumour tissue accumulates K even when muscle-K is less than 60% of normal, as was also found in Sarcoma 180 in K deficient mice [13]. This accumulation of K correlates with the Mg content of tumour (Fig. 6) and is compatible with a high proportion of bound or fixed K in tumour tissue [14]. However, in combined Mg and K deficiency of longer duration there was a significant loss of K from tumours (20%), frequently with gain in Na or Mg and in consequence there was no correlation between tumour Mg and K concentrations. These observations suggest that with increasing Mg and K deficiency there is a gradual transition in tumour tissue from K accumulation, to K loss, with an associated retardation of growth.

Dietary deficiency of Mg and K increases the concentration gradient between extracellular and intracellular fluid in most mammalian tissues. An intracellular depletion of either of these elements is likely to be of greatest consequence in a rapidly metabolising cell and may retard energy producing biosynthetic pathways directly or indirectly by restricting the cell membrane monovalent pump. Cell membrane transport requires the activity of membrane ATPases which are either Mg dependent or Na–K dependent enzymes. However, *in vitro* experiments with Ehrlich ascites tumour cells [15] suggest that it is unlikely that the reduced ATP generation that occurs in K depleted cells is primarily due to inoperability of the Na^+-K^+ transport system. Such cells, with low metabolic activity, have a decreased rate of glycolysis [16, 17] and a low rate of respiration [15, 18], both processes being stimulated by an increase in K. Similar observations have been made with Morris's hepatoma cells [14]. K deficiency may exert its effect on mitochondrial oxidation by reducing the availability of substrate e.g. glutamate [15].

Mg and K depletion causes growth reduction of fast growing tumour cells in the rat. We have not yet attempted to retard growth of an

established slow growing tumour or to show necrosis and tumour regression as was observed in patients [1]. However, it is known that interference with the supply of ATP or with transport systems, as probably occurs in Mg and K depletion, does relate to or result in rapid progression through stages leading to cell death and necrosis [19]. These authors have shown by *in vitro* studies that cell death is related to a fall in cell K, or Mg or ATP. Following the loss of cell K or Mg there is usually an associated uptake of Na^+ (Fig. 3) and probably H^+ and Ca^{2+} which may also have a damaging effect on the cell. Additional anti-tumour effects due to metabolic abnormalities associated with Mg and K deficiency cannot yet be excluded.

In these experiments with rats, three observations were common to those found in patients: (i) Maximum effect on tumour was noted in combined depletion (1); (ii) Correlation between tumour Mg and K and (iii) Difficulty of removing tumour K (unpublished observations). It is suggested that dietary depletion of both Mg and K may affect replication and survival of tumour cells by inhibiting metabolic activity and causing the loss of intracellular K.

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Comparison of Effect of BCG, Glucan and Levamisole on B16 Melanoma Metastases*

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Abstract—*Varying doses of BCG, levamisole or glucan alone, or in combination with different numbers of irradiated tumour cells were given at multiple sites on a single occasion before or after excision of the B16 melanoma growing in the hind limbs of syngeneic C57Bl/6J mice. Facilitation or suppression of spontaneous metastatic spread to the lung was observed with all of the agents studied and depended on the dose of the agent and the time point at which it was administered.*

The result of combining irradiated cells with an immunomodulator could not be predicted from the effects observed when similar doses of these agents were used alone.

INTRODUCTION

"IMMUNOTHERAPY", the practice of attempting to augment the anti-tumour immune response with agents which largely stimulate the reticuloendothelial system, is being increasingly employed in human cancer. Reports on animals and/or man have indicated that treatment with tumour cells [1–3] or treatment with a number of immunomodulators both bacterial, such as BCG [4, 5] and *Corynebacterium parvum* [6], and chemicals such as levamisole [7] and glucan [8, 9] might be useful anti-cancer agents.

To date, most reports on animal models have concerned the effects on local tumours rather than on spontaneous metastatic spread. A few reports involving spontaneous bloodborne metastatic spread [1–3, 7–10] have been moderately encouraging, and since cancer patients die predominantly from metastases such reports must also be considered as more relevant to the problem in man. However, little attempt has been made to compare the effects of one agent with another [6].

We report here on a comparative study of the effects of different doses of three immunomodulators, glucan, levamisole and BCG on bloodborne metastatic spread from the B16 mouse melanoma and the effects of combining various doses of these agents with selected numbers of irradiated tumour cells.

An attempt has been made to assess whether it is possible to predict the effects of combining an immunomodulator with irradiated cells having prior knowledge of their separate effects when used alone.

MATERIAL AND METHODS

Mice

Conventionally housed 8-week-old inbred male C57Bl/6J were obtained from Jackson Laboratories and maintained on rat cake and tap water *ad libitum* in all experiments.

Tumour

The B16 melanoma, which arose spontaneously in the above strain and which has been maintained by long-term transplantation, metastasizes reproducibly to the lung in 100% of animals when grown as a subcutaneous implant in the right hind limb in this laboratory.

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Tumour vaccine

Areas of the leg tumour which appeared most viable were selected and incubated at room temperature for 1 hr in 25 ml of MEM medium containing 13 mg trypsin, 15 mg collagenase and a trace of DNAase (all Sigma Type 1, England) with a magnetic stirrer. The resulting suspension was filtered through gauze to obtain a single cell suspension of greater the 97% viability on phase microscopy. Following overnight incubation at 4°C in MEM medium (Grand Island Biological Co., U.S.A.) containing 10% syngeneic mouse serum the cells were irradiated on a conventional X-Ray machine at 800 R/minute to a level of 15,000 R and 2×10^5, 2×10^4 or 2×10^3 cells injected in 0·05 ml volumes into each of five sites (i.p. and i.d. to four limbs)/mouse.

BCG

Doses of 2·5, 25 and 250 μg of lyophilized viable BCG (Institut Microbiologie et Hygiene strain, Montreal) in a 0·25 ml volume of saline were injected in divided doses of 0·05 ml, into each of five sites (i.p. and i.d. to four limbs)/mouse for groups of 11 mice on a single occasion.

Glucan

Doses of 2·5, 25 and 250 μg of glucan (obtained from Dr. DiLusio, Tulane University, U.S.A.) were injected as for BCG.

Levamisole

Doses of 2·5, 25 and 250 μg of levamisole (McNeill Laboratories, Canada) were injected as for BCG and glucan.

Immunomodulators and irradiated cells

The doses of agents outlined above were injected into five sites as above in 0·05-ml volumes containing either 4×10^5 or 4×10^3 tumour cells/ml.

Experimental design

A total of 10^3 tumour cells were injected into the right hind limb of 726 mice which were divided into 62 experimental groups of 11 and a control group of 44 mice. Half of the experimental groups were treated on Day 8, and the remainder on Day 19. The tumours were excised, by disarticulation of the femoral head of the tumour bearing limb, from 6 mice of each group on Day 18, and 5 on Day 19, as the number of mice in the experiment rendered it impossible to remove all the tumours on the same day. The tumour bearing and contralateral hind limbs were weighed to the nearest 0·1 g and the values for the latter subtracted from the former to give an approximate estimate of the tumour weight.

Analysis of experiments

Thirty one days after tumour excision the mice were exsanguinated under ether anaesthesia, the lungs removed and the metastases (mostly pigmented) counted macroscopically. There were no significant differences in the leg tumour weights or the numbers of metastases found within groups amputated on Day 18 compared to those amputated on Day 19, and the data are tabulated together.

The results were analysed using the Mann Whitney non-parametric test and values of $P < 0{\cdot}05$ were considered significant. The predicted mean number of metastases following treatment with a combination of immunomodulator and autovaccine was compared retrospectively with the observed mean number of metastases in these groups on the basis of the formula below.

Let the mean number of metastases following autovaccine alone = X and the mean number of metastases following treatment with an immunomodulator = Y.

Predicted number of metastases = $(X+Y)/2$.

Agreement within 2 metastases/mouse was considered arbitrarily to represent an accurate prediction and would represent neither an antagonistic nor a synergistic effect. Antagonistic effects would result in observed values of $> (X+Y)/2$ while synergistic effects would be represented by values of $< (X+Y)/2$. Significant antagonism was considered to have occurred when observed values reached $X+Y$, and synergism when values reached $(X+Y)/4$.

RESULTS

Groups of 11 mice were treated with irradiated cells and/or immunomodulators, and the weight of the primary tumour (grams wet weight) and the incidence and number of macroscopic lung metastases compared to those in the 44 controls.

No significant alterations in leg tumour weights were observed, the average for the control groups being 1·19 g while the range for experimental groups was 0·94–1·48 g (Table 1).

At autopsy, a few mice (up to two in any one group) were observed to have tumour recurrence at the site of excision; these were excluded from the analysis. The incidence of metastases in the controls was 97% and although in one experimental group, it was as

Table 1. *The weight of leg tumours following treatment with immunomodulators (BCG, levamisole and glucan) alone or in combination with irradiated B*16 *tumour cells. (Average weight (grams wet weight) of leg tumours (range in brackets))*

Immuno-modulator	Dose (μg/mouse)	No DXR tumour cells	10^4 DXR tumour cells	10^5 DXR tumour cells	10^6 DXR tumour cells	10^7 DXR tumour cells
None		1·19* (0·74–1·85)	0·98 (0·72–1·24)	1·21 (0·87–1·40)	1·41 (0·94–1·69)	0·96 (0·83–1·41)
BCG	2·5	1·19 (0·81–1·75)	1·31 (0·86–1·61)	—	1·12 (0·78–1·37)	—
	25	1·03 (0·76–1·34)	1·01 (0·73–1·21)	—	0·97 (0·70–1·13)	—
	250	0·99 (0·71–1·43)	0·94 (0·68–1·23)	—	1·13 (0·80–1·29)	—
Levamisole	2·5	1·27 (0·91–1·49)	1·16 (0·84–1·33)	—	1·38 (0·77–1·66)	—
	25	1·20 (0·82–1·30)	1·09 (0·77–1·18)	—	0·99 (0·73–1·27)	—
	250	1·36	1·24 (0·93–1·37)	—	1·36 (0·91–1·59)	—
Glucan	2·5	1·20 (0·79–1·49)	0·99 (0·74–1·21)	—	1·48 (0·92–1·78)	—
	25	1·12 (0·71–1·27)	0·97 (0·70–1·32)	—	1·11 (0·78–1·26)	—
	250	1·28 (0·83–1·54)	0·95 (0·69–1·25)	—	1·06 (0·71–1·18)	—

*Average of 44 animals. Remainder of groups consisted of 11 animals.
DXR ≡ Irradiated.

low as 50%, these differences were not considered significant (Table 2).

The average number of macroscopic metastases/mouse in the control groups was 9·2 and a statistically significant degree of suppression or facilitation of metastatic spread occurred in a number of experimental groups (see below).

The effect of irradiated tumour cells (Table 3)

When treated prior to tumour excision, a progressive reduction in metastatic spread occurred as the number of irradiated cells increased from 10^4 to 10^7 and the values for treatment with 10^6 and 10^7 cells were statistically significant.

A similar reduction occurred when mice were treated following tumour excision, except in the group immunized with 10^7 cells in which there was a significant increase in metastatic spread when compared to the controls.

Table 2. *Incidence of spontaneous metastatic spread following treatment with immunomodulators (BCG, levamisole and glucan) alone or in combination with irradiated B*16 *tumour cells. (Average number of lung metastases/mouse)*

		No DXR tumour cells		10^4 DXR tumour cells		10^5 DXR tumour cells		10^6 DXR tumour cells		10^7 DXR tumour cells	
Immuno-modulator	Dose μg/mouse	Pre-excision	Post-excision	Pre-excision	Post-excision	Pre-excision	Post-excision	Pre-excision	Post-excision	Pre-excision	Post-excision
None			40/41	9/10	9/10	11/11	9/10	6/9	7/10	5/11	9/9
BCG	2·5	8/9	10/11	10/11	11/11	—	—	9/10	11/11	—	—
	25	9/9	9/11	11/11	11/11	—	—	7/10	9/11	—	—
	250	8/10	9/11	8/10	10/11	—	—	8/10	7/10	—	—
Levamisole	2·5	8/9	10/10	9/11	9/10	—	—	8/10	9/10	—	—
	25	6/11	8/9	9/11	9/11	—	—	7/11	11/11	—	—
	250	9/9	7/10	6/11	11/11	—	—	6/9	11/11	—	—
Glucan	2·5	9/11	9/10	10/11	8/10	—	—	8/10	10/11	—	—
	25	11/11	9/9	9/10	5/11	—	—	5/11	9/10	—	—
	250	10/11	8/9	9/10	7/11	—	—	10/10	11/11	—	—

DXR ≡ Irradiated.

Table 3. The number of spontaneous metastases following treatment with immunomodulators (BCG, levamisole and glucan) alone or in combination with irradiated B16 tumour cells. (Average number of lung metastases/mouse (range in brackets))

Immunomodulator	Dose μg/mouse	No DXR tumour cells		+10⁴ DXR tumour cells		+10⁵ DXR tumour cells		+10⁶ DXR tumour cells		+10⁷ DXR tumour cells	
		Pre-excision	Post-excision	Pre-excision	Post-excision	Pre-excision	Post-excision	Pre-excision	Post-excision	Pre-excision	Post-excision
None			9·2 (0–49)	9·4 (0–31)	7·8 (0–26)	4·5 (1–7)	4·8 (0–16)	3·0* (0–7)	3·2* (0–11)	2·4‡ (0–9)	25·4§ (1–96)
BCG	2·5	5·2 (0–14)	19·0§ (0–42)	10·2 (0–38)	26·0§ (0–72)	—	—	4·8 (0–17)	6·8 (1–16)	—	—
	25	3·2* (1–5)	6·0 (0–37)	31·8§ (4–79)	16·6§ (2–49)	—	—	9·3 (0–68)	9·0 (0–16)	—	—
	250	4·0* (0–11)	14·6 (0–39)	6·6 (0–16)	8·0 (0–33)	—	—	4·6 (0–16)	3·3* (0–11)	—	—
Levamisole	2·5	2·7† (0–7)	15·1 (1–78)	2·7† (0–8)	12·3 (0–39)	—	—	2·9† (0–11)	16·7§ (5–48)	—	—
	25	1·5‡ (0–6)	10·2 (0–37)	9·2 (0–48)	2·6† (0–8)	—	—	6·2 (0–25)	9·1 (3–17)	—	—
	250	27·7§ (3–37)	1·7‡ (0–5)	0·9‡ (0–4)	15·4 (3–71)	—	—	3·3* (0–12)	3·4* (0–12)	—	—
Glucan	2·5	2·4‡ (0–7)	11·0 (0–23)	3·9* (0–14)	4·2* (0–24)	—	—	2·7† (0–10)	7·1 (0–19)	—	—
	25	15·1 (3–69)	15·5 (3–31)	9·0 (0–27)	4·5* (0–34)	—	—	1·8‡ (0–10)	8·7 (0–23)	—	—
	250	6·1 (0–21)	3·2* (0–9)	6·3 (0–16)	6·4 (0–24)	—	—	18·5§ (4–61)	14·2 (6–19)	—	—

*$P < 0.05$
†$P < 0.01$
‡$P < 0.001$
} significant suppression v controls
§$P > 0.05$ significant facilitation v controls
DXR ≡ Irradiated.

The effect of non-specific adjuvant therapy (Table 3)

A significant reduction in metastatic spread resulted from treatment prior to tumour excision with either 2·5 μg of glucan, 25 μg or 2·5 μg of levamisole or 25 or 250 μg of BCG. In contrast treatment with 250 μg of levamisole produced a significant degree of facilitation, while other treatment schedules resulted in no detectable effects. Treatment with 250 μg of glucan or 250 μg of levamisole after surgical removal of the leg tumour resulted in a significant suppression of metastatic spread while treatment with 2·5 μg of BCG produced a significant increase in the number of pulmonary metastases. Other treatment schedules neither significantly facilitated nor suppressed metastatic spread.

The effect of combining immunomodulators with irradiated tumour cells (Tables 3, 4 and 5)

A significant reduction in metastatic spread resulted from pre-operative treatment with 10^6 irradiated tumour cells combined with either 25 μg or 2·5 μg of glucan and 250 μg or 2·5 μg of levamisole. Similar results followed an injection of 10^4 irradiated tumour cells combined with 2·5 μg of glucan, 250 μg and 2·5 μg of levamisole. A significant degree of facilitation resulted in only two groups, those treated with 25 μg of BCG and 10^4 irradiated tumour cells or 250 μg of glucan and 10^6 irradiated cells.

Following post-operative treatment with a combination of 10^6 irradiated tumour cells with 250 μg of levamisole or 250 μg of BCG resulted in a decrease in the number of metastases, while a significant degree of facilitation occurred on combination with 2·5 μg of levamisole. In groups treated with 10^4 irradiated cells post-operatively, suppression of metastases resulted from combination with 25 μg and 2·5 μg of glucan and 25 μg of levamisole while an increased number of metastases occurred following combination with 25 μg and 2·5 μg of BCG.

An attempt was made to assess the predictability of combining immunomodulators with irradiated cells on the basis of the results

Table 4. Retrospective analysis of predicted v observed results

Difference between predicted and actual number metastases	% of total number of groups (36 groups)	% of number of groups with suppression of metastases (12 groups)	% of number of groups with facilitation of metastases (5 groups)	% of number of groups with no alterations in metastatic spread
0- 2·0	30·6	16·7	0	47·3
2·1- 4·0	19·4	16·7	0	15·8
4·1- 6·0	11·1	16·7	0	10·5
6·1- 8·0	11·1	33·3	0	0
8·1–10·0	5·6	0	20·0	5·3
10·1–12·0	8·3	0	20·0	10·5
12·1–14·0	8·3	8·3	40·0	0
14·1–16·0	0	0	0	0
16·1–18·0	2·8	8·3	0	5·3
18·1–20·0	0	0	0	0
20·1–22·0	0	0	0	0
22·1–24·0	2·8	0	20·0	5·3
Total	100·0	100·0	100·0	100·0

Table 5. Detailed breakdown of analysis for synergistic or antagonistic effects of immunomodulators and irradiated tumour cells

Treatment	Pre operative			Post operative			Total		
	$\left(< \frac{x+y}{4}\right.$	$\simeq \frac{x+y}{2}$	$\left. > x+y\right)$	$\left(< \frac{x+y}{4}\right.$	$\simeq \frac{x+y}{2}$	$\left. > x+y\right)$	$\left(< \frac{x+y}{4}\right.$	$\simeq \frac{x+y}{2}$	$\left. > x+y\right)$
B.C.G.	0/6	4/6	2/6	0/6	2/6	4/6	0/12	6/12	6/12
Levamisole	3/6	3/6	0/6	1/6	3/6	2/6	4/12	6/12	2/12
Glucan	2/6	3/6	1/6	2/6	2/6	2/6	4/12	5/12	3/12
10^4 irradiated cells	3/9	5/9	1/9	3/9	3/9	3/9	6/18	8/18	4/18
10^6 irradiated cells	2/9	5/9	2/9	0/9	3/9	6/9	2/18	8/18	8/18
Total	5/18	10/18	3/18	3/18	6/18	9/18	8/38	16/36	12/36

obtained by treatment with the immunomodulator or the autograft alone.

Thus, (Table 4), the mean numbers of metastases observed from the combination of specific and non-specific therapy are compared to the numbers which might have been predicted from the results obtained following the relevant groups treated specifically or non-specifically alone. A difference of less than two metastases/mouse between expected and observed values was considered arbitrarily to denote an accurate prediction of a negligible effect (e.g., neither antagonistic nor synergistic). Out of a total of 36 therapeutic attempts, a significant reduction in metastatic spread occurred in 12 groups and facilitation of metastatic spread in five groups. Accurate predictions (a difference of less than two metastases/mouse between expected and observed values) could have been made for 2/12 (16·7%) of the groups in which suppression might have been expected compared to 0/5 (0%) for those in which facilitation might have been expected and 8/19 (47·3%) in which no effect might have been predicted. An overall accurate prediction might have been made for 10/36 (27·8%) of all groups.

On the basis of the arbitrary formulation (see Methodology) antagonistic effects were seen in 12/36, synergistic effects in 8/36 and no alteration in 16/36 groups undergoing combination therapy indicating no obvious pattern overall (Table 5). Nevertheless, antagonistic effects occurred more readily following combination with BCG than with glucan and levamisole, and following combination with 10^6 than with 10^4 irradiated tumour cells. Furthermore, such effects occurred more commonly post-operatively than pre-operatively (Table 6).

DISCUSSION

Statistically significant differences in the numbers of lung metastases compared to the control animals were observed in a surprisingly high number of experimental groups.

We feel that this may reflect the fact that immunization was carried out at fairly optimum time points in order to either prevent metastases by immunization at 8 days or to influence existing metastases by immunization at 20 days.

The groups immunized at 8 days constituted an attempt at immunoprophylaxis, since metastases do not develop in these experiments until 15–18 days after implantation (Proctor, unpublished observations) and thus some 7 to 10 days after immunization. In the other half of the experiment the tumours were removed prior to immunization at a time point when the size of individual metastases was minute, and immunity in the bloodstream has been shown to return within 7 days of B16 tumour excision despite the presence of lung metastases (Proctor *et al.*, in preparation).

The effects of irradiated tumour cells were similar to those described previously for other models [1–3] in which metastatic spread varied with the number of tumour cells used for immunization and the amount of tumour present.

Thus very large numbers of irradiated tumour cells may facilitate metastatic spread while more moderate numbers may suppress this event [3], as in the case of the present experiment. Furthermore as the tumour burden increases the number of cells producing facilitation decreases (Proctor *et al.*, in preparation).

The reasons why immunization should facilitate rather than suppress tumour spread are not established. However, one of the most widely held concepts relating to the success of tumours in the face of a detectable immune response resolves around the so-called" blocking mechanisms". These are believed to include effects mediated by antibody, antigen, or antigen/antibody complexes. Immunization with irradiated tumour cells will probably provoke a rise in soluble antigen levels, as described for non irradiated tumour cells [11] due to the fairly rapid cell death following injection of the tumour [12]. Stimulation of many lymph node areas distant from the primary could be expected to boost the anti-tumour immune products, and it is probable that there is an upper limit. Too many irradiated tumour cells may produce an excess of circulating soluble antigen and encourage blocking of immunity. While more likely to occur in the face of large tumour burdens, since immunity is likely to be weak in these instances, the most devastating effects may be expected following tumour removal in the presence of minimal residual disease. In terms of their anti-tumour effect, similar variations (facilitation or suppression) were observed in the present study following treatment with two of the three immunomodulators used, and appeared to depend on the dose of agent and the amount of tumour present at the time of treatment. For a single dose at a single time point one often appeared superior to the others, but overall, since each of them alone or in combination with irradiated tumour cells produced facilitation and suppression in one dose or another, they cannot be regarded necessarily as quantitatively different in their anti-tumour activity.

This finding of facilitation is in agreement with those made on the subcutaneous growth of a variety of tumours in association with levamisole [13] or BCG treatment in which facilitation was observed [14–16]. Chee and Bodurtha [16] showed that the effect of BCG on the growth of subcutaneous B16 melanoma was dose dependant. The reasons for potentiation of tumour growth by the non specific agents are obscure. Either immunostimulation such as that described by Prehn [17], or antigenic competition as described by Liacopoulos *et al.* [18] are possible explanations for the findings made with BCG.

The facilitation observed with 250 μg levamisole in a preoperative group is less easy to explain, as this compound is not an immunogen. A toxic effect could be considered, except that facilitation did not occur in other groups treated with the same dose, which is less than 1/10 of the LD 50 in our experience (Proctor, unpublished observations). Facilitation was not observed with glucan alone, and in this respect glucan differed from levamisole and BCG. We have found that the effect of BCG varies with the route of administration and the site of B16 tumour growth [19]. Our study, like that of Pimm and Baldwin [20], showed that intravenous administration of BCG produced the best effect on systemic tumour. In man, intravenous administration of BCG is strongly contraindicated as the risk of systemic injection is high in patients who are compromised immunologically. Levamisole, however, reaches high bloodstream concentrations following oral administration, and this agent and glucan, which is broken down finally to glucose, have a definite potential as systemic agents.

The results of combining the non-specific adjuvants with irradiated cells were disappointing in that the effects proved unpredictable. The value of analyses such as those we have described on such small numbers of groups is perhaps questionable. Nevertheless, they do demonstrate that both antagonistic and synergistic effects can occur.

In the past we have reported that a combination of a dose of *C. parvum* which was ineffective on its own with a number of tumour cells known to facilitate metastatic spread, was beneficial [3]. Conversely, in another model a dose of *C. parvum* which was ineffective on its own produced a facilitating effect when combined with a number of irradiated tumour cells known to produce a beneficial effect when used alone (Ghaffar, personal communication), and in this respect the present results are similar.

In summary, the treatment with tumour vaccine or immunomodulators appears to be capable of suppressing or facilitating blood-borne metastatic spread depending on the doses used and the amount of tumour present at the time of treatment. The significance of such findings to man is not known, and it is unacceptable to extrapolate findings made in animal models to human cancer patients. Nevertheless, should man be similar in respect to treatment with tumour vaccine or immunomodulators there is a potential risk. Furthermore, a feature of many groups of cancer patients is their heterogeneity. Thus, the prognosis in melanoma patients is intensely variable and depends on a number of factors. Should immunotherapy of the type discussed in this report depend in man on the dose of the agent used, the amount of the tumour present, the immunocompetence of the host, and the immunogenicity of the tumour as appears to be the case in animals, it is unlikely that a dose of an immunotherapeutic agent which succeeds in one patient will benefit more than a small percentage of the succeeding cases.

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A Late Clonal Evolution of a Human Leukemic Line: Sequential Cytogenetic Studies*

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Abstract—*Cytogenetic evolution* in vitro *of a human leukemic cell line has been followed for more than 4 yr. At the beginning of the establishment the karyotype seems normal with some pseudodiploid cells. Then endomitosis and two chromosome markers are observed in a few percentage of the cell population. Some chromosomes show a partial despiralisation of their distal part. After more than 3 yr in culture these abnormalities are present in every cell, demonstrating a late clonal evolution* in vitro. *Studies performed with controlled heating denaturation technic allow identification of the markers and analyse of structural rearrangements.*

INTRODUCTION

THE *in vitro* cytogenetic evolution of human lymphoblastoid cell lines established from normal or leukemic donors has already been studied [1–4], but contradictory results were obtained. Most authors found no differences between cell lines of normal or abnormal origin. Henle described a lesion which involved a C group chromosome, attributed to Epstein–Barr Virus [5]. More recently, Steel has noticed a late clonal-type evolution in one human lymphoblastoid line [6]. Data reported here are concerned with the cytogenetic evolution of a lymphoblastoid line initiated from a leukemic donor.

MATERIAL AND METHODS

The blood cells from a female patient, Mrs HUE, with an acute myeloid leukemia were originated (T_0) and maintained in 1640 RPMI medium supplemented with 20% fetal calf serum using a previously described technique [7]. After an early period during which cell death predominates, (T_d), a multiplication phase occurs indicating the establishment of the lymphoblastoid line (T_i), followed by the exponential phase of growth (T_e) (Fig. 1).

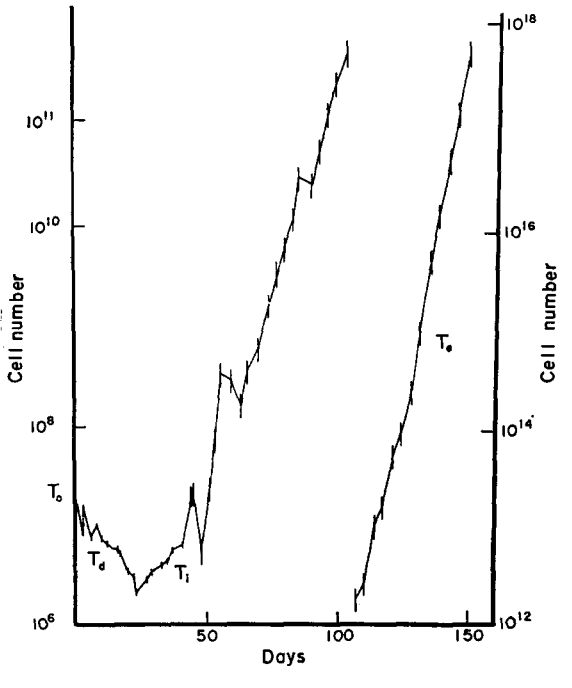

Fig. 1. Curve of the kinetics of the establishment of the HUE lymphoblastoid cell line; T_0*—seeding into culture,* T_d*—death phase,* T_i*—first cell multiplication phase,* T_e*—exponential phase of growth.*

The first cytogenetic study was done three months and 10 days after T_0 (seeding time). The other samples were examined at T_i and at various time intervals during the exponential growth phase (T_e). For each experiment 0·5 to 1 $\times 10^7$ cells were withdrawn from culture

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flasks and treated by conventional techniques for chromosome analysis [8, 9]. The technique of controlled heating denaturation was also used [10].

For each sample studied, 50–100 metaphases were photographed. From the chromosome counts we were able to establish distribution histograms. Twenty-five or more karyotypes were studied for each sample using both classic and denaturation techniques when possible.

RESULTS

When examined 3 months, and 6 months and 25 days, after the initial *in vitro* seeding of the culture (T_0), a normal female karyotype was observed in most of the cells, although some cells were psuedodiploid, showing a loss or gain of one or several elements. Using only the classical method for studying chromosomes, we noted that these abnormalities were obviously non systematized. Nevertheless, their relatively high frequency when compared to normal controls allowed us to exclude the possibility of a technical artefact being responsible.

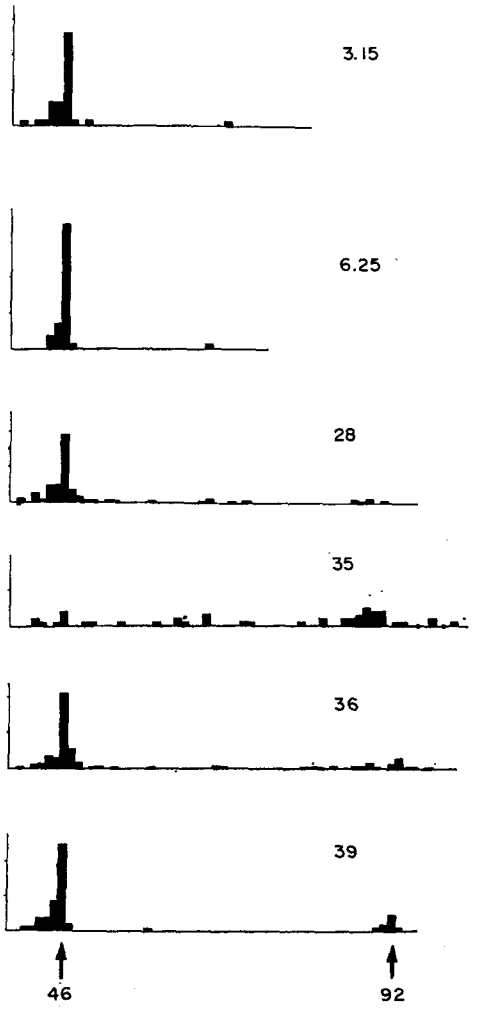

Fig. 2. Histogram depicting the distribution of chromosome number from cell samples of the HUE line at different times in culture. Numbers—months and days.

A third examination, using the same techniques and made 28 months after T_0, showed the following facts.

The number of chromosomes was found to range from hypodiploidy to hypotetraploidy (Fig. 2) with a diploid modal maximum. Numerous endomitosis were observed. Ten karyotypes from diploid cells were analysed. One C and one F elements were missing, but two chromosomes, one of which could be a Cq+ and the other either a Fp− or a Fq− were present (Fig. 3). In one of the 10 karyotypes a slight despiralisation of the end of the arms of chromosome 3 was seen. In another karyotype a despiralisation of the end of the short arms from a B element was observed. At least in several cells, an accentuation of the secondary constriction of chromosomes 1 and 16 (Table 1 and Fig. 4) was also noted. At the 4th and 5th examinations, 35 months and 3 days, and 35 months and 8 days, the respective chromosome numbers had clearly shifted towards pseudotetraploidy (Fig. 2). All the cells contained the elements previously described as Cq+ and Fp− or Fq−. The terminal despiralisation of chromosomes 3 and B was again observed (Fig. 5). Nine days later, an aliquot of the culture was frozen and stored. The remaining cells were carried on in culture and 15 days later analysed again. The same data were found but with an increase in the frequency of 3 and B chromosome terminal despiralisation. Two other samples were studied at 36 months and at 36 months and 4 days. The abnormalities already described were present but the number of pseudotetraploid cells was found to have decreased (Fig. 2). When the controlled heating

Table 1. Table showing the increase of frequency of the accentuation of the secondary constriction of chromosomes 1 and 16

Time in culture	Chromosomes 1		Chromosomes 16	
	On one element	On both	On one element	On both
6 months 25 days	0	0	15	0
28 months	20	0	15	25
39 months	50	35	30	25
47 months	31	69	8	92

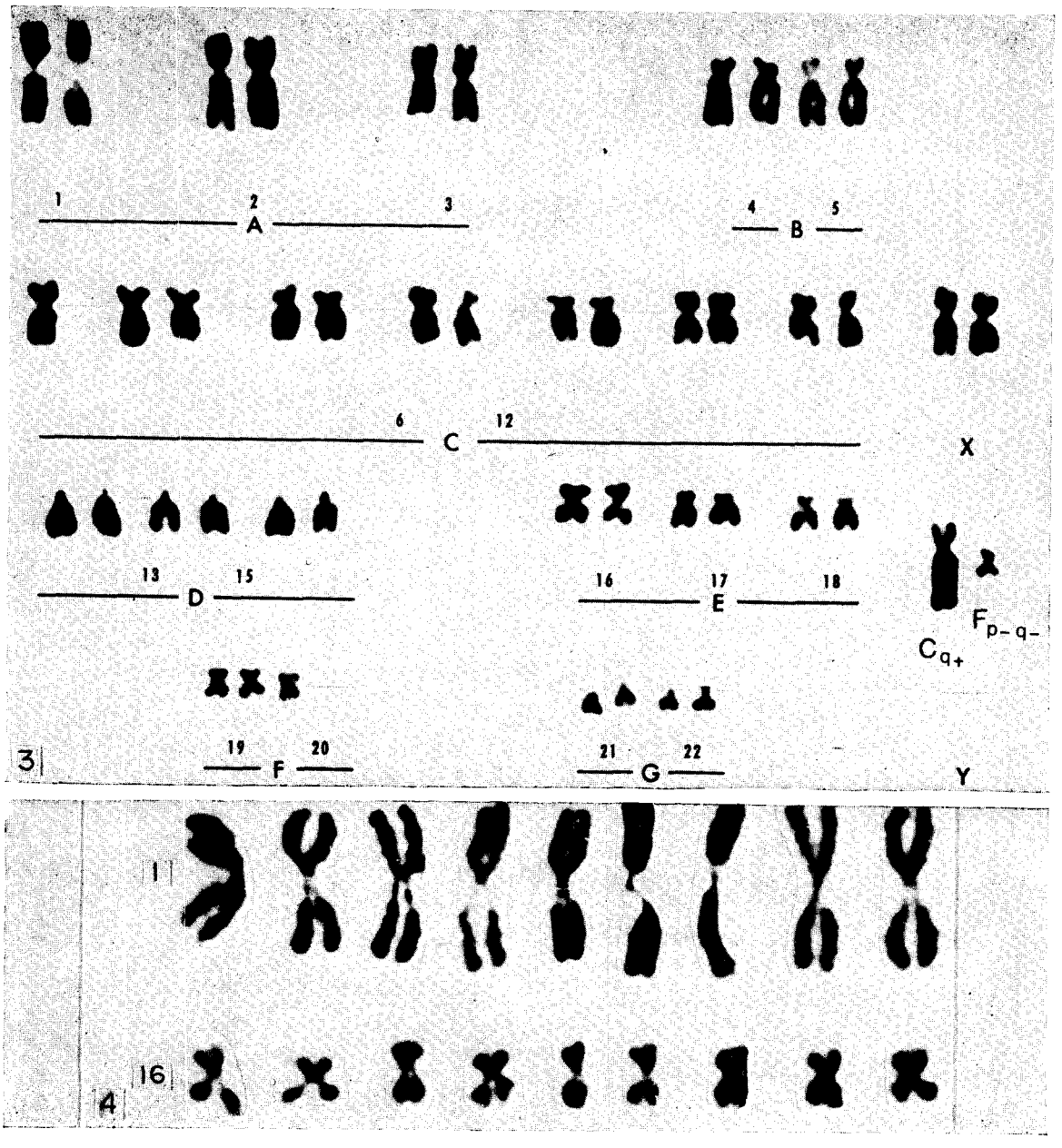

Fig. 3. *Karyotype of HUE line at* 28 *months.*

Fig. 4. *Several chromosomes* 1 *and* 16 *of different mitosis showing an accentuation of their secondary constriction.*

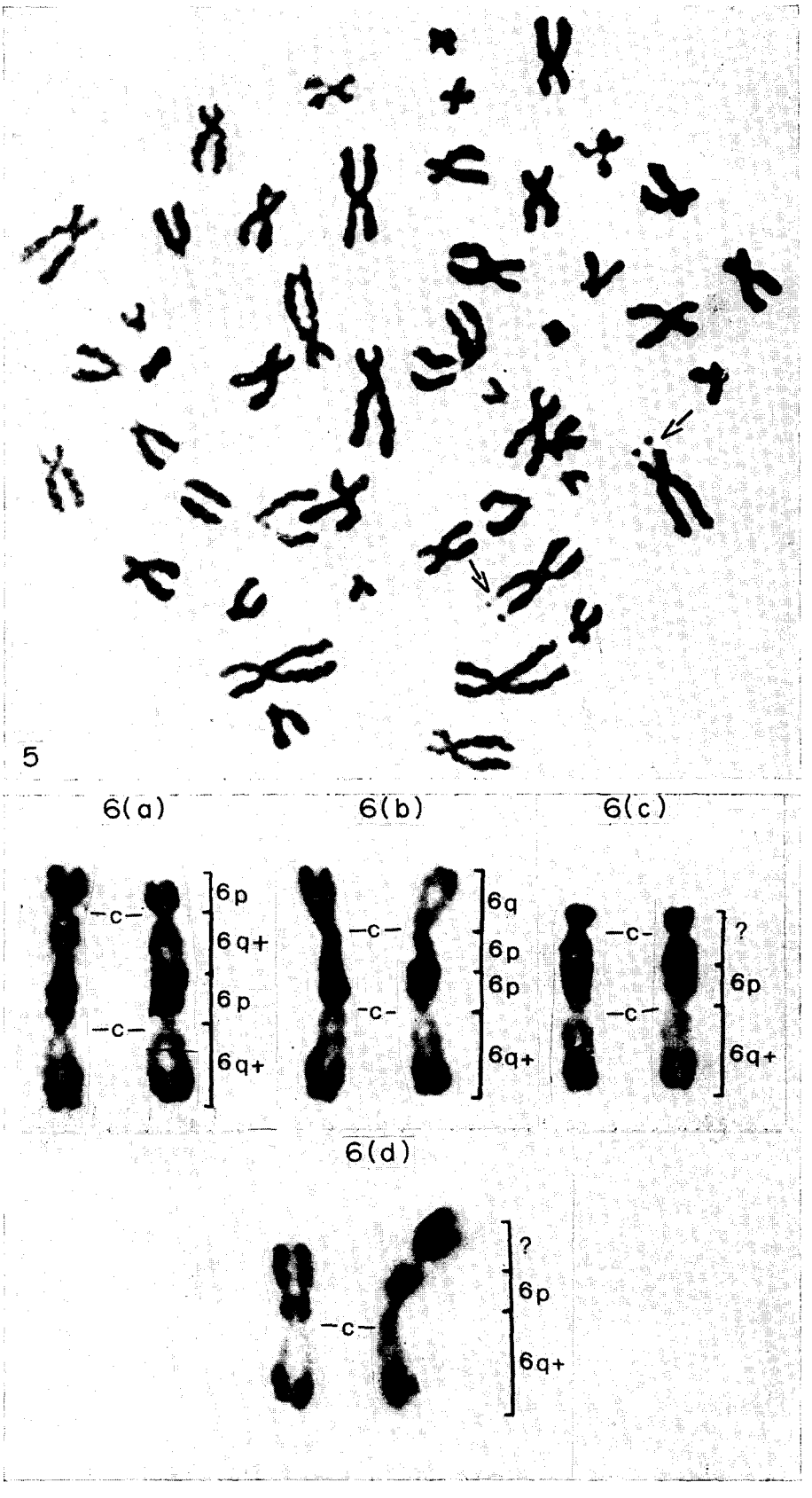

Fig. 5. *Cell metaphase from HUE line at* 35 *months. Arrows show the despiralisation of distal part of the arms of a* 3 and *B chromosome.*

Fig. 6. a.b.c.—*Several dicentric chromosomes found in HUE line cells at* 39 *months. They are partially constituted with* 6 *q*+. d—*great abnormal monocentric element observed in a cell sample after* 47 *months* in vitro.

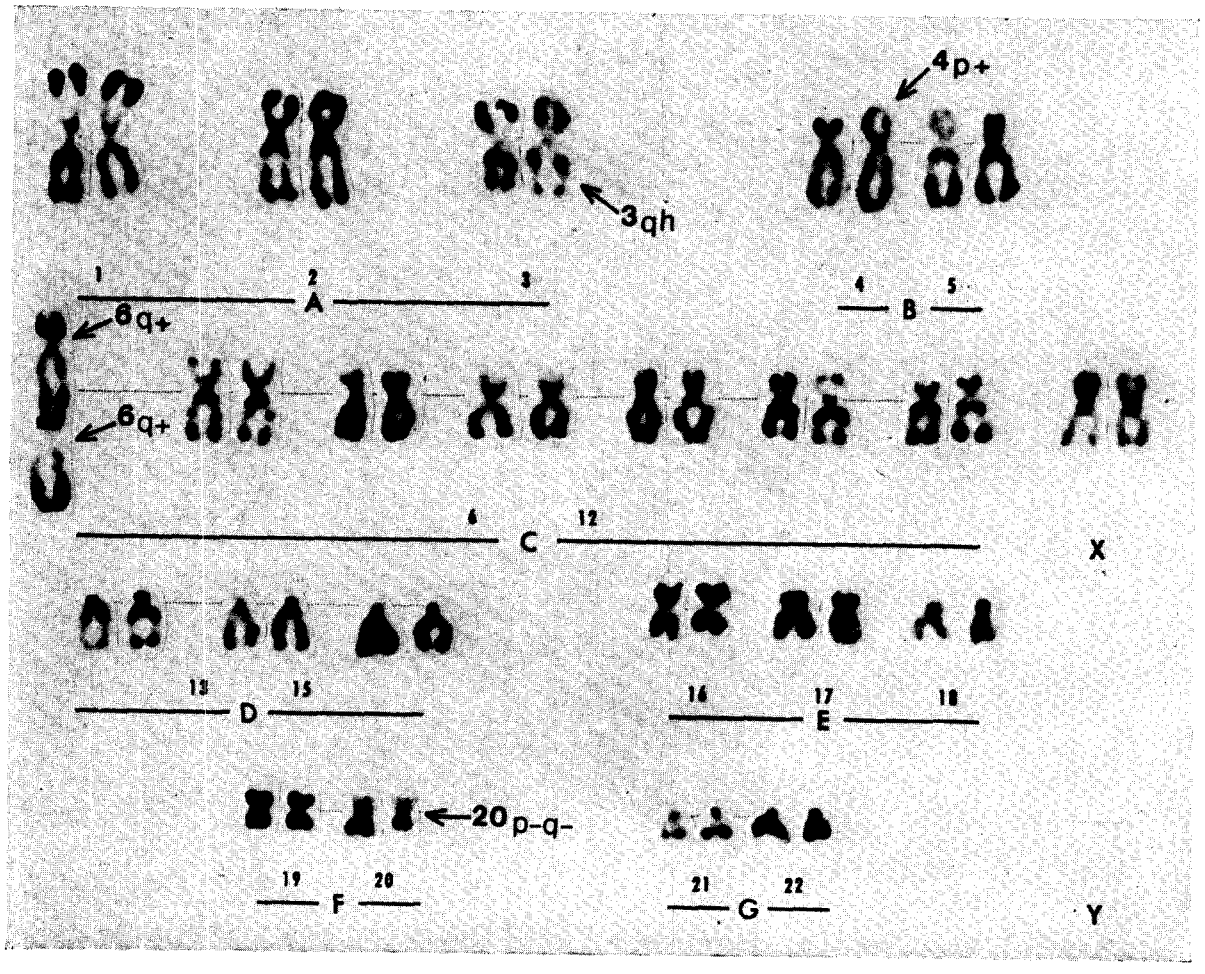

Fig. 7. Karyotype of HUE Cell at 39 months showing the 4 markers: 3 qh, 4 p+; 6 q+ (forming a dicentric chromosome) and 20 p−q−.

denaturation technique was applied to a sample of this culture 39 months and 3 days after T_0, the number of chromosomes had decreased towards diploidy (Fig. 2) with a small fraction of the population being tetraploid. In all cells, one or two 6 chromosomes and one 20 chromosomes were absent. When a 6 element was missing, it was always replaced by a 6q+ as proved by banding techniques. When the two 6 were missing, they were replaced either by one or two 6q+. A dicentric element was found in a few cells. The chromatid analysis with banding showed a partial constitution with a 6q+ and in one case with two 6q+ (Fig. 6a, b, c). In all cases, the presence of a dicentric is counter-balanced by the absence of a 6 chromosome or a 6q+ so that, for the number 6 pair, the total material is roughly comparable to normal diploid cell. Likewise, a small metacentric 20p−q− took the place of a missing 20.

The despiralisation of number 3 chromosome was observed either on the short or on the long arm and sometimes on both. It is placed on the p24 and q26 band according to the Paris classification.

The despiralised B chromosome was a number 4 and the incriminated band was the p16 on the short arm (Fig. 7).

Compared to the preceding observation a higher frequency of the secondary constriction with a lengthening of number 1 and 16 chromosomes was found at 28 months in culture (Table 1).

After 47 months *in vitro*, the controlled heating denaturation technique showed that the previously described abnormalities (3ph, 3qh, 3phqh, 4ph, 6q+ and a 20p−q−) were remarkably stable. Genuine dicentric elements were not found again but some great monocentric chromosomes could be observed. Their banding pattern was very similar to that of the dicentric found at 39 months. The presumed site of one of the centromeres was indicated by a constriction of an apparently secondary nature (Fig. 6d). In many cells an increase in the frequency of secondary constriction with a lengthening was observed on homologues (Table 1). Moreover, in many cells one or several elements were missing without evident systematisation. These abnormalities were less frequent than previously and this suggests karyotype stability. Finally, the sample frozen at 35 months was thawed at 50 months and again maintained in culture. Four months later, i.e. 39 months after the beginning of the culture, the abnormalities already described were again found: 3ph, 3qh, 3phqh, 4ph, 6q+, 20p−q−. In another cell a dicentric element partially composed of a 6 chromosome was also observed.

DISCUSSION AND CONCLUSION

The great variation in distribution of the chromosome number in this leukemic cell line is the first noticeable fact. After an initial diploid period, cells become heteroploid at 35 months. Then, at 39 months they return to a diploid state with a certain number of tetraploid cells. The transition to a tetraploid state, which could be due to endomitosis, does not result in any modification of the abnormalities observed at 28 months. At this time distal despiralisations appear on number 3 and B chromosomes. The number of mitoses affected by this phenomenon increases and involves all the cells at 39 months. We also observed an increasing frequency in the secondary constrictions of number 1 and 16 chromosomes, while the 9th pair was never affected. This phenomenon has already been reported by Zur Hausen [11] and it seems to be correlated more with the duration of the lines *in vitro* than with their normal or leukemic origin, for we also found it in normal cell lines.

The controlled heating denaturation technique enabled us to identify the chromosomes affected by structural rearrangements and to demonstrate that these late appearing markers remain stable for many generations (19 months for 6q+).

One can see that after freezing and thawing, the cells followed the same evolution within the same timespan, independent of the length of conservation.

The problem of the presence of dicentric chromosomes, which were seen for several generations, has already been discussed [12]. This dicentric element always occurs with a 6q+ and seems to be the result of chromatides joining together affording the separation of the two initial constituent chromosomes in anaphase.

Study of the HUE line since its establishment demonstrates a "trying of brushing type" evolution by malsegregation which does not lead to karyotype stabilisation, whereas, in comparison, an evolution by structural rearrangements leads to a stable karyotype. This clonal-type evolution affords a means of stabilisation and survival for the line. Steel proposed that such an event should be used as a criterium for the establishment of a cell line, which, until now, was determined by growth curves [6, 7].

Late clone constitution with marker chromosomes demonstrates a sequence of morphological transformation different from that described by study of growth curves for the establishment of such line. The formation of this late clone must be taken into account when looking for properties which can differentiate between lymphoblastoid cell lines according to their normal or leukemic origin.

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Desensitization of Effective Anti-Tumour Immunity in Guinea Pigs*

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Abstract—*The immunotherapeutic effects of the methanol extraction residue (MER) of BCG were investigated in strain 2 guinea pigs injected with cells of the transplantable, diethylnitrosamine-induced, line 10 hepatocarcinoma. MER was more effective in mediating tumour regression in guinea pigs obtained from the Weizmann Institute of Science, Rehovot, Israel than in animals obtained from the National Institutes of Health. Repeated skin testing with solubilized tumour antigen of MER-treated animals whose tumours had regressed led to recurrent tumour growth and/or susceptibility to rechallenge in six of nine guinea pigs so treated. These results suggest the existence of minimal residual disease even in animals which respond positively to immunotherapy.*

INTRODUCTION

BOTH BCG and the methanol extraction residue (MER) fraction [1] of BCG are non-specific stimulants of cellular and humoral immunity [2–4], and have been successfully employed in tumour immunotherapy in a number of animal systems [5–7]. The tumour model we have been studying is the inbred strain 2 guinea pig diethylnitrosamine-induced, transplantable line 10 hepatocarcinoma [8]. Previous workers have shown with this model that injection of viable BCG directly into growing tumour nodules, at seven days following intradermal implantation of 10^6 tumour cells, leads to regression and cure in approximately 60% of animals so treated [9, 10]. We attempted to duplicate these findings with MER, a substance which offers the advantages of being both non-living and more readily quantifiable on a weight basis than the intact organism [11]. In addition, we investigated the effects of repeated skin testing with solubilized preparations of line 10 tumour antigen on guinea pigs whose tumours had regressed following immunotherapy.

This communication describes experiments showing that the effectiveness of MER as an immunotherapeutic agent for the line 10 hepatocarcinoma was dependent on the substrain of guinea pigs used for experimentation. Furthermore, repeated skin testing with soluble tumour antigens of animals which had apparently been cured by immunotherapy of line 10 disease led to tumour re-appearance followed by death in a substantial proportion of cases.

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MATERIAL AND METHODS

Animals, tumour cells and immunotherapy

Sewall–Wright inbred strain 2 guinea pigs were obtained from the breeding colonies of the Laboratory Aids Branch, Division of Research Services, National Institutes of Health (NIH guinea pigs) and the Weizmann Institute of Science, Rehovot, Israel (WI guinea pigs). WI strain 2 animals were derived from the NIH colony in 1970.

The ascites form of the line 10 hepatocarcinoma was kindly provided in the fourteenth transplant generation by Dr. B. Zbar, National Cancer Institute, Bethesda, Md., and passaged i.p. at least four times in each of NIH and WI guinea pigs before use. Tumour cell suspensions for intradermal (i.d.) challenge were prepared immediately before use as described previously [12], and adjusted to a concentration of 10^7 hepatoma cells per ml of Hanks' balanced salt solution (BSS). Animals were injected i.d. into the right flank with 10^6

tumour cells in 0·1 ml BSS. After 7 days, experimental animals were inoculated with MER (0·5–1·0 mg in 0·1 ml BSS) (kindly supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.) directly into developing tumour nodules. Tumour-injected guinea pigs not receiving immunotherapy served as controls. Female animals only were used in these experiments.

Soluble tissue extracts and delayed cutaneous hypersensitivity (DCH) studies

Soluble tissue components were prepared by 3 M KCl extraction from both ascites-grown line 10 tumour cells (SA-10) and from perfused normal adult liver tissue (SA-N) by a modification of the procedure of Meltzer *et al.* [13] as previously described [14], and stored at −20°C until use. Protein concentrations were determined by the method of Lowry *et al.* [15]. These extracts were used in DCH studies in both MER-treated and control tumour cell-injected guinea pigs. Skin testing was also carried out with purified protein derivative (PPD) of *Mycobacterium tuberculosis*, kindly supplied by the Ministry of Agriculture, Weybridge, England. All skin test inocula were administered in 0·1 ml of 0·15 M NaCl, and areas of induration measured after 24 and 48 hr.

Lymphocyte stimulation

Blood from strain 2 guinea pigs were drawn into heparin by cardiac puncture, and the mononuclear fraction purified by Ficoll–Isopaque gradient centrifugation [16]. This fraction, which consisted largely of lymphocytes, was collected by aspiration, washed twice by centrifugation for 15 min at 500 *g* in BSS and the cells resuspended in bicarbonate-buffered medium RPMI (supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin) to a final concentration of 10^6/ml. Cultures containing 1 ml of cell suspension in 17 × 100 mm tubes were incubated in the presence or absence of various test antigens (i.e. 100 μg SA-N; 100 μg SA-10) for 72 hr at 37°C. Tritiated thymidine (1 μCi/tube; New England Nuclear, Boston, Mass.) was added to the culture tubes for the final 16 hr of incubation, following which the samples were processed by trichloroacetic acid precipitation on to filter pads and the amount of incorporated radioactivity determined.

RESULTS

Comparison of MER immunotherapy effectiveness in guinea pigs derived from different breeding nuclei

The results of a preliminary experiment indicated that five of a group of 14 WI strain 2 guinea pigs were cured of developing line 10 skin tumours by intra-tumour inoculation of 0·5 mg MER at 7 days following implantation of 10^6 tumour cells. These five animals were subsequently all found to be resistant to id challenge with 10^7 tumour cells, 2 months after the initial implantation event, indicating the development of tumour transplantation immunity. These same animals were still alive 14 months later, and showed no trace of recurrent malignant disease. Although the rate of success following immunotherapy with MER was less than that which had been previously reported for living BCG [9, 10], it was decided to investigate this subject further, and to employ guinea pigs derived from different breeding nuclei. The results (Table 1) confirmed that MER could apparently be used to eliminate tumour growth in 18 of 46 or approximately 40% of the animals of WI origin developing line

Table 1. *Effect of MER on line* 10 *hepatoma growth in guinea pigs of NIH and Weizmann Institute origin*

Group	Animal origin	Treatment	No. in group	No. of survivors	Average day of death of non-survivors
1	NIH	0·5 mg MER	12	2	65
2	NIH	1·0 mg MER	12	2	70
3	NIH	—	15	0	71
		% Experimental animals responding = 16%			
4	Weizmann	0·5 mg MER	23	9	75
5	Weizmann	1·0 mg MER	23	9	79
6	Weizmann	—	8	0	64
		% Experimental animals responding = 39%			

10 tumours. A much smaller proportion (i.e., 16%) of NIH-derived pigs, however, responded to the same treatment ($P < 0{\cdot}01$; χ^2 test). All animals with growing tumour died of widespread metastatic disease between 60–90 days after line 10 hepatocarcinoma implantation. By contrast, successful MER immunotherapy generally led to complete tumour disappearance by 3–4 weeks after treatment.

Desensitization of effective anti-tumour immunity by skin testing

The 18-MER treated WI guinea pigs of the experiment of Table 1 whose tumours had regressed were divided into two groups of nine animals each. One such group was skin tested weekly for four consecutive weeks commencing seven weeks after tumour implantation with 0·1 ml of each of SA-10 (100 μg protein), SA-N (100 μg protein), PPD (2 μg protein) and isotonic saline. The second group of nine MER-treated animals were similarly skin tested with SA-N, PPD and saline; SA-10 challenge was omitted. All skin test injections were administered by the i.d. route. Areas of induration were measured after 24 and 48 hr. None of the animals in either group responded to SA-N or saline, while 16 of 18 had delayed cutaneous hypersensitivity (DCH) reactions on at least two occasions against PPD. All 9 guinea pigs of the first group showed strong DCH reactions to SA-10 following each weekly skin test challenge. We further observed that four of the nine animals in the first group, as opposed to none of nine in the control group, developed regional lymph node metastatic tumours and eventually died. Two of these tumours appeared between 3–4 weeks after the commencement of skin testing while the others did not become palpable until after 6 weeks (Table 2). These findings suggested that skin testing with soluble tumour antigen had in some way desensitized the anti-tumour immune response and permitted tumour recurrence.

Table 2. Effect of repeated skin testing with soluble tumour antigen (SA-10) and other antigens on MER-treated, apparently tumour-free guinea pigs

Group	Skin tested with	Animal	Time of appearance of metastatic tumour (days following onset of skin testing)	Date of death (days following initial tumour cell injection)
1	SA-10 (100 μg)	1	24	108
	SA-N (100 μg)	2	27	115
	PPD (2 μg)	3	43	135
	NaCl (0·15 M)	4	49	121
		5	—	147
		6	—	159
		7	—	—
		8	—	—
		9	—	—
2	SA-N (100 μg)	10	—	—
	PPD (2 μg)	11	—	—
	NaCl (0·15 M)	12	—	—
		13	—	—
		14	—	—
		15	—	—
		16	—	—
		17	—	—
		18	—	—

Animals 5–18 were re-challenged with 10^7 tumour cells 102 days following initial tumour implantation.

In order to test this possibility, the five survivors of group 1 and the nine animals of group 2 were challenged i.d. with 10^7 tumour cells as described above, 102 days following initial tumour cell implantation. Two of the five guinea pigs of group 1, and none of the nine of group 2 developed growing tumours at the site of reinoculation and eventually succumbed (Table 2). Thus, skin testing with high concentrations of soluble tumour antigen (1 mg/ml) apparently caused recurrent tumour growth and susceptibility to rechallenge in 6 of 9 MER-treated guinea pigs which otherwise would apparently have remained disease-free ($P < 0{\cdot}001$; χ^2 test).

We also studied the effect of repeated skin testing of these animals on immune responsiveness to SA-10 and SA-N as measured by lymphocyte blastogenesis. This technique has previously been employed as a monitor of specific anti-tumour immunity for the line 10 system [17]. Guinea pigs were bled 10 days following final skin test challenge and 10^6 lymphocytes, obtained by Ficoll–Hypaque gradient centrifugation, were incubated in 1 ml of medium RPMI containing 10% fetal calf serum and various test antigens for 72 hr at 37°C. Tritiated thymidine was added to the culture tubes for the final 16 hr of incubation, and the samples processed as described above. The results (Table 3) demonstrated that most of the guinea pigs tested which had survived tumour challenge following immunotherapy with MER had demonstrable levels of cellular anti-tumour (i.e., anti SA-10) immunity by this technique. Incorporation of label in response to SA-N was generally below background. No differences in either levels or frequency of responsiveness were observed, between that group of animals which had been skin tested with each of SA-10, SA-N and PPD and that group which received SA-N and PPD only. Thus, effective desensitization of anti-tumour immunity, indicated by tumour recurrence and susceptibility to rechallenge following repeated skin testing, could not be correlated with decreased levels of such immunity as measured by standard *in vitro* testing procedures.

DISCUSSION

These data indicate that repeated skin testing with solubilized tumour antigens can apparently cause the recurrence of neoplastic growth in apparently tumour free animals which had previously been treated at a primary tumour growth site with MER. In addition, we have shown that MER is a more effective immunotherapeutic agent for the transplantable line 10 hepatocarcinoma when used in WI than in NIH guinea pigs. This report thus complements a previous study [18] in which it was shown that MER effectiveness in the

Table 3. *Effect of repeated skin testing with SA-10 on SA-10-induced ^{3}H-TdR incorporation by circulating lymphocytes**

Animal No.	History of animal	Skin tested weekly for 4 weeks with:†	Counts/min following stimulation with: NaCl (0·15 M)	SA-N (100 μg)	SA-10 (100 μg)
1	Normal control	—	3759¶	3441‖ NS	2998 NS
2	Normal control	—	3337	2557 NS	3246 NS
3	MER immunotherapy "cure"	PPD (2 μg); SA-N (100 ug); NaCl (0·15 M)	1531	717 NS	1651 NS
4	MER immunotherapy "cure"	PPD (2μg); SA-N (100 μg); NaCl (0·15 M)	2803	1716 NS	4274** $P < 0·01$
5	MER immunotherapy "cure"	PPD (2 μg); SA-N (100 μg); NaCl (0·15 M)	1987	1676 NS	2518 $P < 0·05$
6	MER immunotherapy "cure"‡	PPD (2 μg); SA-N (100 μg); SA-10 (100 μg); NaCl (0·15 M)	2086	1889 NS	2983 $P < 0·01$
7	MER immunotherapy "cure"‡	PPD (2 μg); SA-N (100 μg); SA-10 (100 μg); NaCl (0·15 M)	4397	3947 NS	4986 NS
8	MER immunotherapy "cure"§	PPD (2 μg); SA-N (100 μg); SA-10 (100 μg); NaCl (0·15 M)	1484	1257 NS	2090 $P < 0·05$

*assays performed 90 days after inoculation with tumour cells.
†skin tests carried out between 60 and 80 days after inoculation with tumour cells.
‡developed recurrent metastatic tumours following skin testing.
§died following tumour rechallenge.
¶mean of four replicate samples.
‖not significant.
**probability of significant difference from unstimulated culture.

immunoprophylaxis of the line 10 tumour was restricted to guinea pigs derived from breeding colonies other than that of the NIH.

Several possible explanations come to mind with which to explain these experimental discrepancies. First, it is possible that some degree of genetic drift could have occurred between the NIH and WI breeding stocks since 1970, the year of separation, and that NIH animals may possess an inherent lesser reactivity to MER stimulation. Alternatively, histocompatibility antigen differences may have resulted in an increase in antigenicity of line 10 tumour cells for animals of either NIH or WI origin, thus contributing to the success of BCG or MER immunotherapy in these respective guinea pigs populations. Indeed, should histocompatibility differences exist, then at least part of the tumour rejection phenomenon which both we and others [9, 10] have observed may be due to homograft rather than tumour immunity. If this is the case, however, it is nonetheless obvious that the histocompatibility differences must only be minor, since tumour inoculation in the absence of immunotherapy has been invariably fatal for the animals we have studied. For this reason, it is fair to speak of anti-tumour immunity in this system. These difficulties may be intrinsic to many or all tumour transplantation models. Another explanation for the observed differences is suggested by the recent findings of several groups [14, 19, 20] that BCG cross-reacts antigenically with line 10 tumour cells. Conceivably, injection of substances like MER or BCG may, in some substrains more than others, stimulate the formation of specific antibodies or cellular immune elements, capable either of bestowing heightened resistance or enhancing tumour growth [21]. This finding of shared antigenicity with BCG may also explain the superior results others have obtained in the immunotherapy of the line 10 tumour with either living BCG or BCG walls in admixture with mineral oil.

The mechanism responsible for observed tumour recurrence in the desensitized, MER-treated guinea pigs remains obscure, but it is possible that repeated skin testing with SA-10 may have led to the formation of tumour growth enhancing antibodies [22]. Alternatively, specifically antigenic SA-10 molecules may have found their way into the circulation and interfered with effective humoral and/or cellular immunity [23, 24]. Our results also suggest the apparent survival of residual numbers of viable tumour cells for as long as 4 weeks after disappearance of a palpable tumour mass. These cells are apparently present in the form of micrometastases in the regional lymph node, which was the site of tumour recurrence in each instance studied.

On the basis of an antigen-dependent lymphocyte blastogenesis assay, we were not able to differentiate immunologically between those skin tested guinea pigs which succumbed to recurrent tumour growth and those which survived. It is especially noteworthy that the guinea pigs in our experiment showed DCH reactivity to SA-10 following each skin testing event. This points to the difficulties involved in attempting to prognosticate from the results of either *in vitro* or *in vivo* testing procedures, and complements earlier results obtained with this tumour model [17, 25].

It is not known whether similar results would have been obtained had repeated skin testing been performed with lower concentrations of soluble tumour antigen (e.g. 10 μg which also elicits positive DCH responsiveness). In addition, it is conceivable that 3 M KCl extracts of tumour tissue, as opposed to extracts obtained by other procedures, may contain components which are especially enhancing to neoplastic growth. Finally, since BCG is apparently more effective than MER in the immunotherapy of this tumour, treatment with the former agent may more successfully eliminate residual viable tumour cells.

Nevertheless, these results suggest the continuing existence of minimal residual disease in at least some animals which respond positively to cancer immunotherapy. While such minimal residual disease may frequently be held in check by the animal's defense mechanisms, experimental manipulation can apparently upset this delicate balance and lead to recurrent tumour growth. As such, these results raise questions as to the safety of repeated skin testing with human tumour antigens in a clinical setting.

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Changes in Nuclear RNA Transport Incident to Carcinogenesis*

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Abstract—*Rats were treated with the hepatocarcinogens thioacetamide and dimethylnitrosamine and the release (transport) of RNA from the isolated liver nuclei to homologous or heterologous liver cytosol was evaluated in a cell-free system at various times after treatment. Within 24 hr of treatment, cytosol from the carcinogen-treated animals enhanced the release of RNA from liver nuclei of untreated rats. This enhanced transport capacity of the cytosol persisted up to 4 months after treatment; furthermore, the RNA transport from the liver nuclei of carcinogen-treated animals showed a partial loss of its ATP-dependence. Although the capacity of cytosol from treated animals to support RNA transport dropped below control levels by 9 months after treatment, the RNA transport from nuclei of the carcinogen-treated animals remained partially ATP-independent. This ATP-independence, which is also a characteristic of nuclei from hepatomas, is not a characteristic of the age of the animal, nor is it due to differences in the pool size of nuclear ATP or the requirement for polyadenylation of messenger RNA for transport.*

INTRODUCTION

ACCORDING to current theory both messenger and ribosomal RNA are derived from large nuclear precursors by non-conservative processing [1, 2]. There is also accumulating evidence that this nuclear processing and/or nucleocytoplasmic transport of the processed RNA are under post-transcriptional controls. For example, the proportion of potential messenger, or ribosomal RNA reaching the cytoplasm varies during development [3], with growth rate [4] and upon neoplastic transformation [5]. The limitations imposed on the study of the regulation of RNA processing and transport in the intact cell, has led to the development of cell-free systems which support these processes [6–11]. One of these systems developed in this laboratory to study nucleocytoplasmic controls, shows a dependence of ribosomal and messenger RNA release as ribonucleoprotein particles from either normal, or tumor cell nuclei, on macromolecules in the cytosol [7, 8, 12]; some cytosol-dependence has been observed by other investigators [11, 12]. However, other cell-free systems release their nuclear RNA to incubation media of different composition in the absence of cytoplasmic macromolecules [6, 9].

Despite the apparent differences in cytosol-dependence, both types of cell-free systems exhibit an ATP-dependence of RNA release when the nuclei are derived from normal liver [6–8, 13–15]. In contrast, liver nuclei from the liver of donor animals treated with the hepatocarcinogens dimethylaminoazobenzene, acetylaminofluorene, or thioacetamide was first reported by Smuckler and coworkers [15–17] to show a significant loss of their ATP-dependence. The release of RNA from myeloma cell nuclei was also reported [10] to be ATP-independent. In order to resolve the apparent discrepancies between ATP-dependence in normal liver nuclei and independence in myeloma cell nuclei (i.e. 2 cell types of different origin) a comparison was made of the ATP-dependence of RNA release from the nuclei of normal rat liver, a differentiated rat hepatoma (Hepatoma 5123D) and an undifferentiated rat hepatoma (Novikoff hepatoma). The RNA release was found [14] to be totally dependent, partially dependent and independent of ATP, respectively, suggesting that ATP-dependence

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is related to the degree of differentiation of the tumor. The present study analyzes the loss of ATP-dependent release of RNA from liver nuclei incident to treatment of the host animals with hepatocarcinogens, using the cytosol-dependent RNA transport system; special emphasis is placed on localizing the cellular site at which ATP-independence develops and the time-course of its development.

MATERIAL AND METHODS

The donors of liver tissue were 200–250-g male rats of the Sprague–Dawley strain (Laboratory) Supply Co., Indianapolis, Indiana) before (controls) or after treatment with the well known hepatocarcinogens thioacetamide (Apache Chemicals, Seward, Illinois) or dimethylnitrosamine (freshly redistilled; Eastman Kodak, Rochester, New York). The carcinogenic regimens involved a course of nine daily injections of 50 mg/kg of body weight of thioacetamide [18] or a single injection of dimethylnitrosamine (5·0 mg/kg of body weight) 24 hr after partial hepatectomy [19]. Although dimethylnitrosamine in particular may produce tumors at other sites (e.g. kidney tumors), when given to partially hepatectomized rats according to the indicated protocol, it induces hepatocellular carcinomas in at least 35% of the rats [19]. The protocols selected for dimethylnitrosamine and thioacetamide, besides inducing hepatomas, are particularly suited to the present study since the treatment is of short duration, allowing one to study the irreversible effects of the drugs after cessation of treatment. The nuclear RNA was prelabeled *in vivo* for 30 min with [6-^{14}C] orotic acid (40 μCi/250 g) prior to removal of the liver for nuclear isolation. Non-specific toxicity of the carcinogens is a minor problem in the present study since most of the measurements were made weeks or months after cessation of treatment. All injections were via the intraperitoneal route and the animals were fasted for 17 hr (overnight) prior to use in order to deplete liver glycogen.

The ascites form of the rapidly growing dedifferentiated Novikoff hepatoma was carried i.p. in 140 g female rats of the Sprague–Dawley strain. After washing with 0·9% saline, the nuclear RNA of the hepatoma cells was prelabeled by incubation (10^7 cells/ml) at 37°C for 20 min in Eagles minimal essential medium (Schwartz–Mann, Orangeburg, New York) containing 2 μCi/ml of [5,6-^{3}H] uridine (S.A. = 39·3 Ci/m-mole). Before homogenization the cells were removed from the incubation medium, washed once with 0·9% saline and once with 2·0 vol of the homogenization buffer.

Nuclei were isolated from liver tissue by homogenization in 15 vol of 2·3 M sucrose, 3·3 mM calcium acetate as previously described [7, 20]. Novikoff hepatoma cells were disrupted by suspension in one volume of homogenizing medium composed of 30 mM sucrose, 2·0 mM $MgCl_2$, 3·0 mM $CaCl_2$, 10 mM Tris–HCl (pH 8·0), 0·1% Triton X-100, 0·5 mM dithiothreitol [21] followed by 20 strokes of a tight-fitting Dounce homogenizer. The nuclei were purified by layering the homogenate over 20 ml of 2·0 M sucrose, 3·3 mM calcium acetate and centrifuging for 60 min at 34,000 ***g***. After washing in 1·0 M sucrose-1·0 mM calcium acetate (300 ***g*** for 5 min), the nuclei were resuspended in the same buffer for addition to the cell-free transport system.

Liver cytosol (105,000 ***g*** supernatant) which contains RNA transport factors was prepared from a 1:2 homogenate and dialyzed at 4°C for 18 hr against TMK buffer (50 mM Tris–HCl, pH 7·5, 2·5 mM $MgCl_2$, 25 mM KCl) as previously described [7, 8]. Cytosol was similarly prepared from the Novikoff hepatoma by swelling the washed cells for 10 min at 0°C in 2 volumes of TMK buffer, centrifuging out the cells, followed by a Dounce homogenization (20 strokes with a tight pestle) of the cell pellet. The resulting homogenate was centrifuged, dialyzed against TMK buffer and frozen until use. The protein concentration of the dialyzed cytosol was determined by the Biuret method [8].

The cell-free system which was used to study the release of labeled messenger-like RNA consisted [8] of approximately 5×10^6 prelabeled nuclei/ml of medium containing 12 mg dialyzed cytosol protein/ml, 50 mM Tris–HCl (pH 7·5), 25 mM KCl, 2·5 mM $MgCl_2$, 0·5 mM $CaCl_2$, 0·3 mM $MnCl_2$, 5·0 mM NaCl, 2·5 mM Na_2HPO_4, 5·0 mM sperimidine, 2·0 mM dithiothreitol, an energy source and 300 μg/ml of yeast RNA. Where indicated the energy source, composed of 2·0 mM ATP, 2·5 mM phosphoenolpyruvate and 35 units/ml of pyruvate kinase, was omitted from the reaction. The mixture was incubated at 30°C for 30 min, or for the specified time interval. During the initial 30 min of incubation over 80% of the labeled nuclear RNA released to the medium is messenger-like [8, 20, 22].

The amount of RNA release from the nuclei was estimated as follows. After incubation, the assay mixtures were chilled on ice, and then centrifuged at 1000 ***g*** for 10 min to remove

the nuclei. RNA was precipitated by the addition of 1/10 volume of 50% trichloroacetic acid. Following a wash with cold 95% ethanol the precipitate was dissolved in solubilizer for radioassay in liquid scintillant [20].

Alternatively when a measure of the proportion of the labeled transported RNA containing poly (A) tracts was desired, the RNA was purified from the nuclei-freed incubation medium by phenol:chloroform (1:1) extraction as previously described [22]. The RNA was then passed through a column of oligo-dT-cellulose (Collaborative Research Inc., Waltham, Mass.) to separate the poly A containing RNA, which binds to the column in 0·5 M NaCl, from the RNA lacking poly A, which does not bind under these conditions [23].

RESULTS

Effect of hepatocarcinogens on RNA transport

Preliminary to studying the prolonged (irreversible) effects of the hepatocarcinogens on the energy-dependence of RNA transport, the acute effect of these agents was investigated using the cell-free system described by Schumm and Webb [8]. In these experiments, the livers of the carcinogen-treated rats were tested 24 hr after a single injection of dimethylnitrosamine [19] or the last of 9 daily injections of thioacetamide [18]. The specific dosages are given under Material and Methods.

Figure 1 shows the acute effect of (a) thioacetamide or (b) dimethylnitrosamine treatment on RNA transport. The release of RNA from normal liver nuclei to normal liver cytosol (i.e. the control) is shown for comparison; note that the transport of labeled RNA essentially ceases after 30 min of incubation. The release of [^{14}C]-RNA from the nuclei to homologous cytosol prepared from liver 24 hr after cessation of treatment with thioacetamide was depressed to within 25% of the control value after 30 min incubation.

Thioacetamide-induced liver damage is observed despite the fact that the specific activity of the nuclear RNA is equivalent in the control and treated rats. In contrast, the release of messenger-like RNA from normal liver nuclei is markedly enhanced by incubation in medium containing cytosol from thioacetamide-treated rats. This enhancement, which amounted to 165% of the control value at 30 min incubation, was not observed when cytosol was prepared from an animal 2 hr after a single injection of thioacetamide (M. Hanausek-Walaszek, unpublished observations).

Similarly within 24, but not 2, hr of dimethylnitrosamine treatment (Fig. 1b), there is a 230% increase in the capacity of cytosol to support RNA release from normal liver nuclei during the 30-min incubation. However, in contrast to thioacetamide treatment, nuclei from dimethylnitrosamine-treated rats release considerably more RNA to either homologous, or control liver cytosol than do control nuclei, the release at 30 min being 250% for homologous cytosol and 175% for control cytosol. This enhanced release is not observed with partial hepatectomy alone. Thus, the nuclear

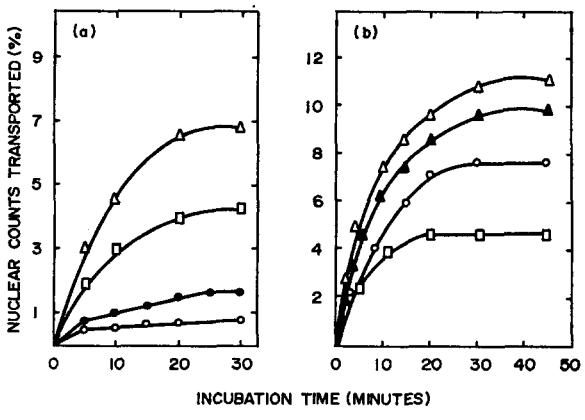

Fig. 1. Acute effect of hepatocarcinogens on the ability of liver nuclei and cytosol to support RNA transport in a cell-free system.

The nuclei were prelabeled for 30 *min* in vivo *with* [6-^{14}*C*] *orotic acid and the rats were examined* 24 *hr after the last dose of carcinogen. In all experiments the cytosol protein was* 10 *mg/ml of assay and the specific activities of the nuclei from normal, thioacetamide and dimethyl nitrosamine-treated rats were* 10,800, 10,120 *and* 8850 *counts/min* 5×10^6 *nuclei, respectively. The amount of labeled RNA transported is expressed as percent of total nuclear counts.* (a) *Normal liver nuclei plus liver cytosol from normal* (—□—) *or thioacetamide-treated rats* (—△—) *and nuclei from thioacetamide-treated rats plus cytosol from thioacetamide-treated* (—●—) *or normal* (—○—) *rats.* (b) *Normal liver nuclei plus liver cytosol from normal* (—□—) *or dimethylnitrosamine-treated* (—△—) *rats and nuclei from dimethylnitrosamine-treated rats and liver cytosol from dimethylnitrosamine-treated* (—▲—) *or normal* (—○—) *rats.*

damage, which may be due to the toxicity of the carcinogens, as estimated by RNA release, is either different from that produced by thioacetamide, or it is repaired within the 24 hr period. The molecular basis for this early enhanced transport is not clear; however, the possibility that it is due to increased nuclease activity was ruled out since there was no change in the soluble counts and the addition of exogenous ribonuclease inhibitor prepared from rat liver (Searle Diagnostic, Arlington Heights, Illinois) did not decrease the amount of RNA transported. Furthermore, none of the RNA

Table 1. Temporal changes in the capacity of cytosol from hepatocarinogen-treated rats to support messenger RNA release from nuclei of normal (control) liver

Cytosol derived from liver of	Percentage of normal liver homologous system at 2 hr	1 day	5 days	1 month	2·5 months	4 months	9 months
Thioacetamide-treated (9 daily doses, 50 mg/kg)	82*	175	155	157	200	162	81
Dimethylnitrosamine-treated (one dose, 24 hr after partial hepatectomy, 50 mg/kg)	83	236	248	248	234	200	60

*This rat received a single dose of thioacetamide. Duplicate experiments checked to within 10% in this and subsequent experiments.

release can be attributed to nuclear leakage of the RNA, since essentially all was dependent on the presence of cytosol proteins (M. Hanausek-Walaszek, unpublished observations).

The temporal changes in the transport of messenger RNA in response to thioacetamide or dimethylnitrosamine treatment are summarized in Table 1. The enhanced capacity of cytosol from the carcinogen-treated rats to support [^{14}C]-RNA release from normal liver nuclei is observed as early as one day and persists for at least 4 months (i.e. in Table 1 the transport of RNA from normal liver nuclei of untreated or control rats to cytosol derived from the liver of carcinogen-treated rats is compared to transport from normal liver nuclei to homologous cytosol). The enhancement than falls to below control levels at approximately 9 months after the final treatment. This decline is not attributable to aging, as transport in systems derived from normal rats of equivalent age were similar to those of young adults. Thus approximately 5% of the total nuclear counts were being transported in systems derived from the livers of 9-month-old rats during a 30-min incubation (A. Yannarell, unpublished observations). Note that these changes, observed from several days to months after cessation of treatment, must represent permanent or semi-permanent changes in regulatory mechanisms of the cell rather than to non-specific toxic effects. Also since the animals appeared healthy and any tumors present at time of assay, when present, were extremely small and were excised before processing of the liver, the presence of tumor tissue could not account for the effects observed. It should be emphasized that the main point of these experiments is that the enhanced capacity of the cytosol from carcinogen treated rats to support RNA release persists for at least 4 months but is lost by 9 months.

The data in Table 2 describe the ATP-dependence of RNA transport from the liver nuclei isolated from rats 4 months and 9 months after the cessation of treatment with hepatocarcinogens. As shown previously [14], the release of RNA from normal liver nuclei is essentially ATP-dependent. In contrast, the

Table 2. ATP-dependence of RNA release in homologous and heterologous cell-free systems derived from the liver of normal and hepatocarcinogen-treated rats

Pretreatment of donor liver: Nuclear source	Cytosol source	Percentage of [^{14}C] RNA released from nuclei* 4 months post-treatment +ATP	4 months post-treatment −ATP	9 months post-treatment +ATP	9 months post-treatment −ATP
none	none	4·4	0·1	4·2	0·1
none	thioacetamide	7·0	4·1	3·4	1·8
none	dimethylnitrosamine	9·3	3·1	3·2	1·5
thioacetamide	none	3·9	2·2	—	—
thioacetamide	thioacetamide	6·2	4·9	3·4	1·4
dimethylnitrosamine	none	3·0	1·0	—	—
dimethylnitrosamine	dimethylnitrosamine	8·4	6·3	2·5	0·7

Duplicate experiments checked to within 10%.

*The columns labeled 4 months and 9 months post-treatment refer to the duration between the cessation of treatment with the carcinogen and the removal of the liver for the preparation of the nuclei.

release of RNA from normal liver nuclei to the liver cytosol derived from hepatocarcinogen-treated rats was significantly ATP-independent. The ATP-independence varied from 30% in systems derived from dimethylamine-treated rats to 58% in comparable systems derived from thioacetamide-treated rats, both at 4 months after treatment. From an analysis of transport in the homologous and heterologous systems, it can be concluded that both the nuclei and cytosol contribute to this ATP-independent component. Furthermore, the loss of ATP-dependence does not appear to be fully accounted for by the enhanced capacity of the cytosol to support RNA transport since significant ATP-independent transport exists even beyond 9 months after termination of treatment despite the fact that the amount of [^{14}C] RNA released after 30 min of incubation is significantly lower than normal. (Note that the data in Fig. 1 show the acute affects of the carcinogens on RNA transport while the data in Table 2 show the effects observed 4 or 9 months after treatment.)

Effect of neoplastic transformation on RNA transport

As shown earlier [14], RNA transport in homologous systems derived from the moderately differentiated hepatoma 5123D and dedifferentiated Novikoff hepatoma is partially and completely ATP-dependent, respectively. Table 3 presents data on crossover experiments

Table 3. ATP-dependence of RNA release in homologous and heterologous cell-free systems derived from normal liver and the Novikoff hepatoma

Source of		Percentage of nuclear RNA transported	
Nuclei	Cytosol	+ATP	−ATP
liver	liver	4·4	0·1
hepatoma	liver	4·2	3·5
liver	hepatoma	3·6	0·2
hepatoma	hepatoma	3·1	3·1

The data shown are the average of triplicate experiments; the standard errors were less than 10%.

between liver and the Novikoff hepatoma designed to identify the site of the lesion leading to ATP-independent RNA release. The hepatoma system is simpler to study than the hepatocarcinogen-treated liver system since the uncharacterized cytosol component responsible for enhanced RNA release is not present; in fact, normal liver nuclei release less RNA to cytosol from the Novikoff hepatoma than to cytosol from normal liver. The data in Table 3 clearly show that, in the case of the hepatoma, modification from ATP-dependence to independence which accompanies transformation resides solely in the nucleus; i.e., transport in the liver (nuclei): liver (cytosol) and hepatoma: liver systems is 0·1 and 3·5% respectively, and in liver: hepatoma and hepatoma: hepatoma 0·3 and 3·1%, respectively.

The existence of a pool of ATP in the nucleus of the Novikoff hepatoma cell could produce an apparent loss of ATP-dependent RNA transport. In order to eliminate this possibility, cells were incubated with ^{3}H-uridine for 10 min, followed by further incubation after the addition of 10 mM deoxyglucose, or 2 mM sodium cyanide for an additional 10 min prior to cellular disruption and nuclear isolation. As indicated in Table 4, this procedure did not alter the ATP-independence of the RNA release, although both the total counts in the

Table 4. Effect of cellular ATP-depletion on subsequent RNA release from Novikoff hepatoma nuclei

Pretreatment with metabolic inhibitor*	Percentage of nuclear counts released	
	+ATP	−ATP
none	3·1	3·1
2-deoxyglucose	2·3	2·2
cyanide	2·5	2·4
cordycepin	1·3	1·1

*The intact cells were pretreated with the inhibitors before isolation of the nuclei.

nucleus and the percent released after 30 min of incubation were decreased. This decrease was predictable since the reduction of the cellular ATP pools by cyanide and deoxyglucose would tend to inhibit RNA synthesis.

Under conditions employed in the present experiments (i.e. a 30-min prelabel *in vivo* followed by a 30-min *in vitro* transport) over 80% of the labelled RNA transported resembles messenger RNA since it is released as ribonucleoprotein particles with the density of informosomes [8, 20]. Because the nuclear processing of a significant fraction of the heterogeneous nuclear (pre-messenger) RNA includes the addition of a poly (A) tract to the 3′-end of the molecule [1], it was desirable to determine whether the proportion of transported messenger containing poly (A) tracts was affected by the elimination of ATP from the cell-free system. Therefore, the proportion of labeled poly (A)-containing RNA released

from Novikoff hepatoma nuclei to homologous, or heterologous cytosol in the presence and absence of ATP, was estimated by determining the percentage of transported RNA which bound to an oligo-dT-cellulose column [23]. The results of these experiments are shown in Table 5. Surprisingly, the proportion of transported labeled RNA containing poly (A) tracts was the same in the absence and presence of ATP in both liver and Novikoff cytosols. The fact that there is no reduction in the poly (A) content is consistent with the earlier observation [26] that interference with polyadenylate formation *in vivo* leads to decreased RNA transport *in vitro*. In agreement with this finding, it was observed that treatment of Novikoff hepatoma cells with cordycepin (3'-deoxyadenosine) caused a reduction in the percent of RNA transported but no change in the ATP-independence of the transport (Table 4). The validity of the results of the cordycepin experiments rests on the premise that cordycepin does not interfere with ATP metabolism.

DISCUSSION

Although the release of RNA from cell-free systems derived from the liver of hepatocarcinogen-treated rats clearly acquires a partial but significant ATP-independent component, the results also show an enhanced RNA transport during the first 4 months after treatment and a depressed transport after 9 months. However, when taken together the experimental evidence strongly suggests that a portion, if not all, of the ATP-independent transport observed after treatment with carcinogens is due to a specific lesion in RNA processing and, or transport. There is, however, one observation, not yet explicable, between the lesions(s) which characterizes the loss of ATP-dependence of RNA transport in the liver of hepatocarcinogen-treated rats (i.e. preneoplastic liver) and the Novikoff hepatoma (neoplastic liver). In the former, both the nuclei and the cytosol appear to contribute to the phenomenon, while in the latter, the lesion is clearly localized to the nucleus. The observed loss of ATP-dependence of RNA transport following pretreatment with liver carcinogens are in general agreement with previous studies [15–17], the results of which are discussed below. The results of the present study rule out mere leakage of RNA from the nuclei since the transport in both the liver and hepatoma systems is completely dependent on macromolecules in the cytosol.

As noted above the loss of ATP-dependence of RNA transport, as exemplified by the Novikoff hepatoma, is attributable to an alteration in the nucleus. The fact that there was no decrease in the proportions of messenger RNA containing poly(A)-tracts transported from the Novikoff hepatoma nuclei when ATP (and phosphoenolpyruvate) was omitted from the incubation medium, suggests that in tumor cells the poly(A) tracts may be added to the nuclear pre-messenger RNA much sooner after synthesis than in the normal cell, although a number of other explanations are possible. It does not appear to be due to a large nuclear pool of ATP in the tumor cells. The elucidation of this difference awaits further study.

On the other hand, ATP also appears to be necessary for RNA transport through the nuclear pores of normal liver tissue both *in vivo* and *in vitro* (i.e. in the cell-free system). Thus beryllium nitrate, an inhibitor of nuclear pore phosphatase inhibits messenger RNA release from normal and neoplastic cell nuclei in proportion to their ATP-dependence [14]. For example, RNA transport in the cell-free systems derived from normal liver, Hepatoma 5123D and the Novikoff hepatoma were inhibited approximately 80%, 35% and 2% by 30 mg/ml of beryllium nitrate; the corresponding energy-dependence of RNA transport in these systems are 100%, 25% and 0%, respectively. Together these results suggest that the loss of energy-dependence in the tumor is due to changes at the level of the nuclear pore complex. In this regard, it is of interest that in precancerous liver, cell foci induced by diethylnitrosamine can be identified as adenosine triphosphatase-deficient islands [27, 28]. It is tempting to speculate that this enzyme deficiency and the loss of ATP-dependent transport are related. However, the fact that the adenosine-triphosphate-deficient islands in the livers of carcinogen-treated rats account for less than 1% of the tissue mass [27] makes it unlikely that such islands alone account for the ATP-

Table 5. Proportion of labeled RNA containing poly (A) tracts released from Novikoff hepatoma nuclei to Novikoff and liver cytosol in the presence and absence of ATP

ATP in incubation	Percentage of labeled RNA with poly (A) tracts	
	Novikoff cytosol	Liver cytosol
–	22	19
+	22	18

independent transport observed in the hepatocarcinogen-treated livers. One explanation is that the treatment with carcinogen may lead not only to a complete deletion of adenosine triphosphatase in a few cells which constitute the foci of precursor tumor cells, but rather to a partial reduction in the activity of this enzyme in most of the parenchymal cells and that the latter decrease is not observed by the histochemical test. It is clear that further studies are required to determine whether the loss of this enzyme and the ATP-dependence of RNA transport are related events.

Aside from the locallization of the apparent lesion in tumor cells to nuclear RNA processing and/or transport the ATP-independent transport of RNA from nuclei of the Novikoff hepatoma provides further clues concerning the site of action of ATP. Thus the fact that RNA release from Novikoff nuclei is ATP-independent whether they are incubated in normal liver or hepatoma cytosol, and conversely the ATP-dependence of RNA transport from normal liver nuclei when incubated in either cytosol, rules out the possibility that the ATP-requirement in the normal cell is related to phosphorylation of the cytoplasmic proteins required for RNA transport. The possibility that energy donors other than ATP contribute to this ATP-independent-transport is doubtful. Since the cytosol is dialyzed, such pools, which are obviously absent from normal liver nuclei would have to be sufficient to support RNA transport for 30 min and this despite the loss of nucleotides during nuclear preparation. Furthermore, regeneration of such nucleotides would require ATP.

It is clear that the *enhanced* RNA transport characteristic of cell-free systems derived from hepatocarcinogen-treated rats is due to an increase in the capacity of the cytosol to support RNA transport. Such a change may result from an increase in the positive feed-back, or a decrease in the negative feedback transport factors, both of which have been shown to regulate messenger RNA release in the cell-free system [13]. The modified RNA transport and/or loss of ATP-dependence may account for the defective regulation of hepatic tyrosine transaminase observed after treatment of the rats with dimethylnitrosamine or thioacetamide [29].

The results of the present study relative to the development of ATP-independence of RNA release from liver nuclei following treatment of rats with hepatocarcinogens are in general agreement with the results of Smuckler and coworkers who studied the effect of acute intoxication [16] with thioacetamide; these studies were restricted to the period zero to 72 hr after a single dose of 50 or 200 mg/kg of body weight. These workers observed an initial drop in RNA (RNP) transport and a release of significant portion of RNA from the treated livers in the absence of ATP. In a further study utilizing this protocol, they reported [17] that the acute thioacetamide intoxication was associated with the appearance of more cytoplasmic RNA's (*in vivo*) with migrations of 9–16S, with increases in the proportion containing poly(A) and an enhanced leakiness of the nuclei toward 9S RNA *in vitro*. The *in vitro* studies utilized in modified form the RNA transport system originally developed by Ishikawa *et al.* [6], which in contrast to the system used in the present study does not show cytosol-dependence of RNA release. The RNA release in the Ishikawa system is also much higher than that of Schumm *et al.* (i.e. over 20% cf. 5%). This difference may be partially accounted for by the longer (40 min) *in vivo* labeling period employed [16] which would be expected to result in the transport of considerable labeled ribosomal RNA together with labeled messenger and 4S RNA. It should be noted in this regard that the degree of conversion of heterogeneous nuclear RNA to messenger RNA in some eukaryotic cells may vary from 4 to 20% [30]; furthermore, a large fraction of this labeled nuclear RNA is ribosomal precursor which after a 30-min *in vivo* label, is not transported in our system unless the *in vitro* incubation is extended beyond 30 min [8]. The reason for the difference in cytosol dependence of the two cell-free systems is less clear. Recent evidence from this laboratory indicates that the cytoplasmic transport factors are very specific and in low concentration in the cytosol [22, 31]; they can not be replaced by non-specific proteins such as dialyzed normal plasma [32], or 10 mg/ml of β-macroglobulin or serum albumin (D. E. Schumm, unpublished observations). The system of Schumm *et al.* [8] would appear to be useful for the study of nucleocytoplasmic controls in normal and neoplastic cells; however, both systems have detected changes in ATP-dependence of RNA transport incident to carcinogenesis.

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Effect of Drostanolone Propionate on the Binding of Oestradiol and Dihydrotestosterone by Normal and Malignant Target Tissues*

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Abstract—*The influence of drostanolone propionate, an anticancer agent, was tested on the binding of 17β-oestradiol and dihydrotestosterone to specific receptor proteins in tissue of normal and neoplastic target organs. Steroid binding capacity was measured by agar gel electrophoresis of tissue extracts.*

Drostanolone was found to compete with androgen binding sites but not with oestrogen receptors. Therefore it is unlikely that the growth inhibitory effect of drostanolone propionate in human breast cancer is mediated through interaction with oestradiol binding proteins as suggested earlier by other authors.

INTRODUCTION

DROSTANOLONE propionate (2α-methyl-dihydrotestosterone propionate, Masterid,® Fig. 1) has been reported to be an anti-cancer agent for advanced human breast cancers [1–4] and also for 7,12-dimethylbenzanthracene(DMBA)-induced mammary tumours of the rat [5].

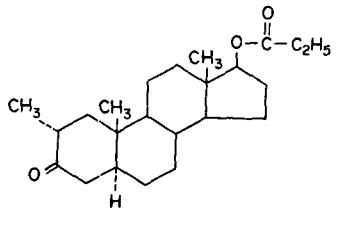

Fig. 1. Structure of 2α-methyl dihydrotestosterone propionate.

Deshpande *et al.* [6] had shown that pretreatment with this compound decreased the uptake of injected ^{3}H-oestradiol in human mammary tumors compared with normal breast or other tissues. Therefore they draw the conclusion that the growth inhibitory effect of drostanolone propionate is mediated through a reduced uptake of oestradiol by the tumor. Under this aspect it was of interest to study the influence of drostanolone propionate on the binding of 17β-oestradiol and 5α-dihydrotestosterone (5α-DHT) to specific cytoplasmic receptor proteins and to the sex hormone binding globulin (SHBG) of the plasma.

MATERIAL AND METHODS

Chemicals

(6,7-^{3}H) 17β-oestradiol (spec.act. 48 Ci/mM) and (1,2-^{3}H)-5α-dihydrotestosterone (spec.act. 49 Ci/mM) were purchased from New England Nuclear Corp., Boston, Mass. The purity (> 94·0%) was checked by thin layer chromatography. The anti-oestrogenic compound U. 11·100 (nafoxidine) was a gift from the Upjohn Company, Kalamazoo, Mich., drostanolone propionate was provided by the Chemie Grünenthal, Stolberg, and cyproterone acetate by the Schering AG, Berlin. All other chemicals were purchased from E. Merck, Darmstadt or Boehringer, Mannheim and were of analytical grade.

Tumour induction

Mammary carcinomas were induced in female Sprague–Dawley rats by a single feeding of 50 mg DMBA at day 50. DMBA was

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dissolved in sesame oil (50 mg/ml). Starting 4 weeks after the carcinogen appearance of tumors was assessed by palpation. Tumor sizes were measured weekly by calipers in the two greatest dimensions (D and d). Tumor mass was calculated by the formula $V = D \times (d)^2/2$ [7]. Ovariectomy in rats was performed at the time when tumors had reached a diameter of at least 2 cm. Those tumors which regressed after removal of the ovaries were called "hormone dependent".

Receptor assay by agar gel electrophoresis

Tissue was frozen in liquid nitrogen immediately after removal and pulverized with the Mikro-Dismembrator (Braun, Melsungen). The fine powder was transferred to a centrifuge tube and four volumes (vol/weight) Tris–HCl buffer (0·01 M, pH 7·5, 1 mM NaN_3) were added. After thawing the sample was centrifuged for 90 min at 40,000 rev/min. (157,000 g_{av}) and 2°C (L2–65B, Beckman Instr.). The supernatant was removed by pipetting and used immediately. Aliquots of the extract were incubated at 4°C overnight with (6·7-^{3}H)-oestradiol or ^{3}H-5α-DHT in presence or absence of radioinert compounds without shaking. The concentrations used are defined in the legends of the figures. At the end of the incubation period, aliquots (50 μl per well) of each sample were subjected to gel electrophoresis, which was performed according to Wagner [8]. Gel layers were prepared with a 1% agar solution (0·05 M Michaelis buffer, pH 8·2). In the centre line of the gel plate, wells were punched out and 50-μl aliquots of the labelled extracts were applied. Two wells were charged with material from one sample. The prepared gel plates were then placed on a teflon coated brass plate within an airtight electrophoresis chamber. The plate was cooled to 2°C. Electrophoresis was carried out for 90 min at 110 mA/300 V. After the run, the gel was divided lengthwise and then cut into 3 mm wide sections. Radioactivity was eluted from the strips with scintillation fluid (7·0 g PPO, 0·3 g dimethyl-POPOP, 100 g naphthalene in 1000 ml dioxane) for at least 4 hr before counting.

Total protein content of tissue extracts was determined according to Lowry *et al.* [9].

RESULTS

In a first series of experiments we tested the effect of drostanolone on the oestrogen binding capacity of normal and neoplastic target organs. Aliquots of cytosols prepared from calf uterus or human breast cancer tissue were incubated with ^{3}H-oestradiol in presence or absence of Nafoxidine or drostanolone propionate. The electrophoretic analyses of the labelled extracts are demonstrated in Figs. 2 and 3. The anodical peak, stretching from the starting line (indicated by the arrow) to fraction 20, represents the receptor bound steroid, while the free hormone is shifted towards the cathode (left side in the figures).

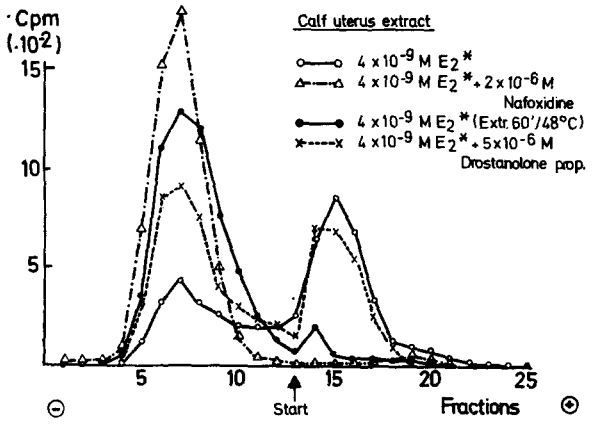

Fig. 2. Determination of oestradiol binding in calf uterus cytosol by agar gel electrophoresis in absence and presence of Nafoxidine and drostanolone propionate, respectively. Protein content of the cytosol was 8·2 mg/ml.

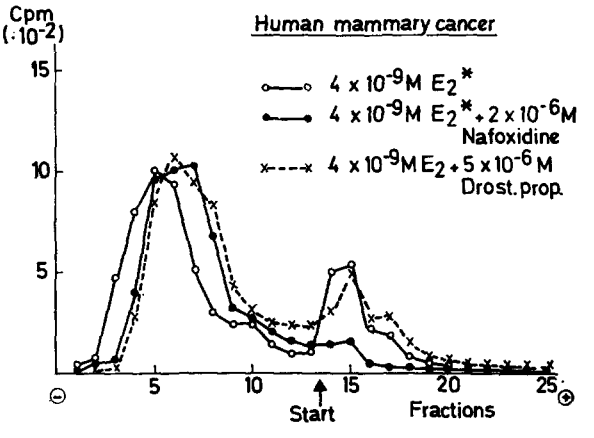

Fig. 3. Determination of oestradiol binding in human mammary cancer cytosol by agar gel electrophoresis in absence and presence of Nafoxidine and drostanolone propionate, respectively. Protein content of the cytosol was 12·4 mg/ml.

Inactivation of the binding protein by heat (60 min at 48°C) or displacement of the labelled oestradiol by the anti-oestrogenic compound Nafoxidine results in a disappearance of the receptor bound labelled hormone and in an increase of the cathodical peak by the liberated steroid. In contrast to this finding drostanolone propionate added in a 1000-fold excess does not compete at the oestrogen-specific binding sites. This holds true both for

calf uterus (Fig. 2) and for human breast cancer tissue (Fig. 3) demonstrated by the persistence of the receptor peak (broken line).

With respect to the fact that drostanolone propionate is a testosterone derivative it was obvious to test this compound on its androgen binding characteristics. As the simultaneous occurrence of oestrogen and androgen receptors was described as well for calf uterus [10] as for human mammary cancer [11–14] the cytosols of these tissues were likewise assayed for their androgen binding capacity. Extracts were incubated with ^{3}H-5α-DHT and analysed electrophoretically in the same manner as described above (Figs. 4 and 5). The 5α-DHT-receptor-complex is characterized by the same mobility as the oestrogen binding protein. The analysis of the tumour extract (Fig. 5) moreover illustrates the clear discrimination between receptor protein and the sex hormone binding globulin (SHBG) from the serum. The last one binds favouredly 5α-DHT and migrates towards the cathode. Addition of a

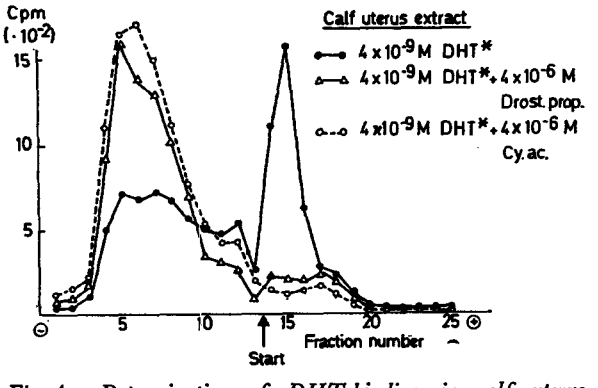

Fig. 4. Determination of DHT-binding in calf uterus cytosol by agar gel electrophoresis in absence and presence of drostanolone propionate and cyproterone acetate, respectively. Protein content of the cytosol was 7·2 mg/ml.

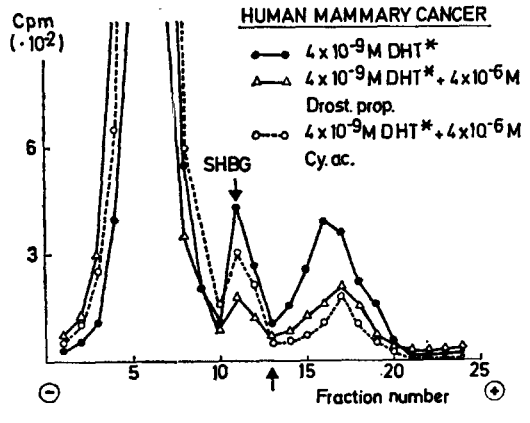

Fig. 5. Determination of DHT-binding in human mammary cancer cytosol by agar gel electrophoresis in absence and presence of drostanolone propionate and cyproterone acetate, respectively. Protein content of the cytosol was 8·0 mg/ml.

1000-fold excess of the anti-androgen cyproterone acetate or of drostanolone propionate results in a decrease of radioactivity at the anodical peak, which is due to competition at specific receptors sites. This competition is less pronounced for SHBG by cyproterone acetate than for drostanolone propionate. Figure 6 shows the electrophoretic pattern of a cutaneous metastasis, which was assayed for oestrogen and androgen receptors. This specimen was derived from a patient with metastatic breast cancer who showed remission after treatment with drostanolone propionate. This medicament

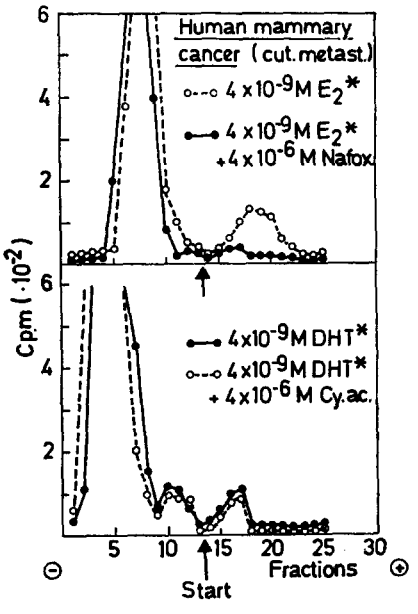

Fig. 6. Determination of oestradiol and DHT-binding in human mammary cancer. Because of cutaneous metastases the patient was treated with drostanolone propionate before excision of metastatic tissue for receptor assay (for further details see text). Protein content of the cytosol was 5·6 mg/ml.

was given (3 × 100 mg/week) over a period of 17 months up to 7 days prior to excision of the tumor. This tumor possesses oestrogen receptors (34 fmole/mg protein), but no DHT-binding is obtained (lower panel). This effect may be due to a primary lack of androgen receptors or it is due to a depletion of spare DHT-receptor sites by the abundance of circulating drostanolone. Kinetic studies [15] have shown, that after a single injection of drostanolone propionate the maximal concentration of free drostanolone in serum remains at a constant level between day 4 and 12 after administration.

DISCUSSION

This investigation was undertaken to obtain some more insight into the mechanism by

which drostanolone propionate exerts its growth inhibitory effect on a variety of mammary tumours. The data presented clearly indicate a competition for androgen binding sites in the cytosols of uterus as well as human breast cancer tissue. In contrast to these findings drostanolone propionate does not affect the oestrogen binding capacity of these target tissues.

Because of these findings it is unlikely that drostanolone propionate exerts its growth inhibitory effect in mammary tumors by competition for specific oestradiol receptor proteins. This is contradictory to the findings of Deshpande *et al.* [6] who noticed a pronounced reduction of 17β-oestradiol in breast cancer tissue after pretreatment with the androgenic compound. Discussing this point it must surely be stressed that the experimental conditions of the studies mentioned were different. Deshpande *et al.* and Braunsberg *et al.* measured the uptake of ^{3}H-oestradiol, which was applicated *in vivo* after pretreatment of the patient with drostanolone propionate. Heise and Görlich and our group determined the specific binding of ^{3}H-oestradiol *in vitro* adding the androgenic compound simultaneously with the labelled steroid. That means that the results obtained by Deshpande *et al.* and Braunsberg *et al.* are comparable regarding the experimental procedures. But in contrast to Deshpande's work the infusion experiments of Braunsberg *et al.* as well as the *in vitro* studies of Heise and Görlich support our results. Both groups likewise could not demonstrate an effect of drostanolone propionate on oestrogen binding in their assay systems.

Recent studies [15] have shown, that drostanolone propionate itself is the active growth inhibitory compound and not a metabolite.

Based on studies on DMBA-induced mammary tumors in rat Van Der Gugten [18] and Hagen *et al.* [19] have suggested that the effect of drostanolone propionate is mediated through a decrease of prolactin secretion by the pituitary gland. Our investigations did not deal with this possibility and it is still open whether the results of these authors are true for human breast cancer.

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Regression of Massive Liver Involvement by Metastatic Breast Cancer after Chemotherapy as Monitored by Scintigrams Using a Stereological Method*

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Abstract—*Six cases of massive liver involvement by metastatic breast cancer who responded to combination chemotherapy are reported. Prior to treatment, positive liver biopsy was obtained in five patients. Liver size was accurately measured by scintigraphy. The relative volume of neoplastic tissue was assessed on the scans by a stereological method and ranged from* 42 *to* 77%. *Improvement of liver involvement under chemotherapy was remarkable both with regard to liver size and relative neoplastic volume which decreased to between* 5 *and* 55% *of the initial value. Control liver biopsies were obtained in four patients and were negative.*

INTRODUCTION

MASSIVE liver involvement by metastatic breast cancer is notoriously resistant to endocrine therapies [1]. Thus Nemoto *et al.* [2] reported that regression occurred in only one out of 13 patients subjected to adrenalectomy or ovariectomy, who had more than one third of the liver replaced by tumour tissue. In contrast, nine out of 21 patients with limited liver involvement responded to these treatments. The use of single-agent chemotherapy has not improved the therapeutic response of liver metastases [3], the remission rate being around 20%. The introduction of multiple drug chemotherapy of breast cancer seems however to have improved the situation [4, 5]. Nevertheless documentation of liver involvement and response to treatment is difficult because there is no irrefutable method of assessment [6, 7]. Although there is a controversy as to the accuracy of liver scanning in detecting liver disease it could provide the most accurate measurements of the liver size, an exact anatomical localization and recognition of filling defect [8, 9].

Our purpose is to report six cases of massive liver involvement by metastatic breast cancer, i.e. replacement of more than one third of the organ by tumour tissue, who dramatically responded to combination chemotherapy. Assessment of response was carried out by means of stereological method of estimation of the relative metastatic tissue volume from liver photoscans.

MATERIAL AND METHODS

Six cases of metastatic breast cancer patients were selected on the basis of their massive liver involvement and most impressive response to combination chemotherapy, as judged by serial scintigrams. No attempt was made at estimating the exact proportion of liver responses by a systematic analysis of all cases treated.

The median age of the patients was 60

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(range 49–78). Biopsy proof of breast carcinoma was obtained in each. All but one (patient No. 5) were postmenopausal. They had widespread disease, always involving both liver and other sites except for patient 5 who had only liver involvement. All but one (patient 1) had undergone surgery and radiotherapy as treatment of the primary. Patient 1 had been subjected to adrenalectomy. All had progressive disease at the time of the study.

The liver status was assessed prior to treatment by clinical examination, liver function tests, liver scanning and liver biopsy under direct visualisation (patient 3 had neither peritoneoscopy nor liver biopsy prior to treatment). In most patients liver function tests were repeated about every 6 weeks after initiation of therapy and liver scanning approximately every 3 months. At least two control scans were performed in the course of treatment except in patient 6, for whom only one was available. Control laparoscopy was carried out and control biopsies were obtained in all cases except for patients 1 and 5.

Patients 1 and 2 received a five-drug combination chemotherapy as described by Cooper [10]. Patients 3–6 received a hormonal–cytotoxic combination chemotherapy that is currently being tried by the European Organization for Research on Treatment of Cancer (E.O.R.T.C.) Breast Cancer Cooperative Group (study protocol 10741; preliminary results reported by Engelsman *et al.* [11]). This chemotherapy comprises tamoxifen (ICI 46474), an antioestrogen, given orally at the dosage of 20 mg twice daily and cytoxic agents given as two alternating cycles of 28 days; first cycle: adriamycin 75 mg/m^2 i.v. on day 1 and vincristine, 1·4 mg/m^2 i.v. on days 1 and 8; second cycle: 5-fluorouracil, 600 mg/m^2 i.v. on days 1 and 8, methotrexate, 60 mg/m^2 i.v. on days 1 and 8 and cyclophosphamide, 100 mg/m^2 orally from days 1 to 15. Patient 4 had dexamethasone in addition.

Liver scintigraphy was performed with a conventional rectilinear scanner. ^{198}Au (150 μCi) was injected i.v. 1 hr before scintigraphy. Relative volume of metastatic tissue was calculated from photoscans by a method derived from the stereological technique described by E. R. Weibel for applications in electron microscopy [12]. For that purpose a regular point lattice was drawn on transparent paper, each point (intersection of two lines) being one cm apart. This lattice, larger than the photoscan, was placed at random on it. A differential count of the points lying on normal (P_n) or pathological images (P_p) was made, the total number of points (P_t) lying on the image being $P_t = P_n + P_p$. From these data, relative volume of metastatic tissue (V_p) is obtained, $V_p = P_p/P_t$. Counting was carried out independently by two of the authors. The results used are the means of the two independent countings. Pathological foci were defined as areas of hypocaptation with at least 24% less activity than the surrounding tissue. This value was established from phantom experiments on the scanner used.

RESULTS

Liver status prior to treatment is described in Table 1. Physical examination revealed a hard and irregular enlargement of the liver in all patients (liver edge palpated from 5 to 20 cm below the costal margin on the midclavicular line). The enlargement was painful in most patients. Direct visualisation by laparoscopy (4 cases) or laparotomy (1 case) and liver biopsy confirmed the physical findings; gross metastatic involvement was present in all. All liver biopsies were positive. Serum alkaline phosphatase and γ-glutamyl trans-

Table 1. *Liver assessment prior to treatment*

Patient number	Liver edge palpated below the costal margin (cm)	Alkaline phosphatase ($N \leqq 85$) (mU/ml)	SGOT/ SGPT ($N \leqq 20$) (mU/ml)	γGT* ($N \leqq 18$) (mU/ml)	Bilirubine ($N \leqq 1$) (mg %)	Relative metastatic tissue volume on scan (%)	Height of right lobe measured on the scan ($N \leqq 16$ cm)	Liver biopsy and direct visualization ((+) if positive)
1	13	200	315/96	—	0·6	51	22	yes (+)
2	15	400	77/79	494	1	75	27	yes (+)
3	20	700	32/22	320	0·8	77	23	not done
4	16	120	32/37	76	3·6	43	21	yes (+)
5	16	250	125/86	—	3·4	56	21	yes (+)
6	5	820	60/32	120	3·6	42	23	yes (+)

*γ-Glutamyl transpeptidase.

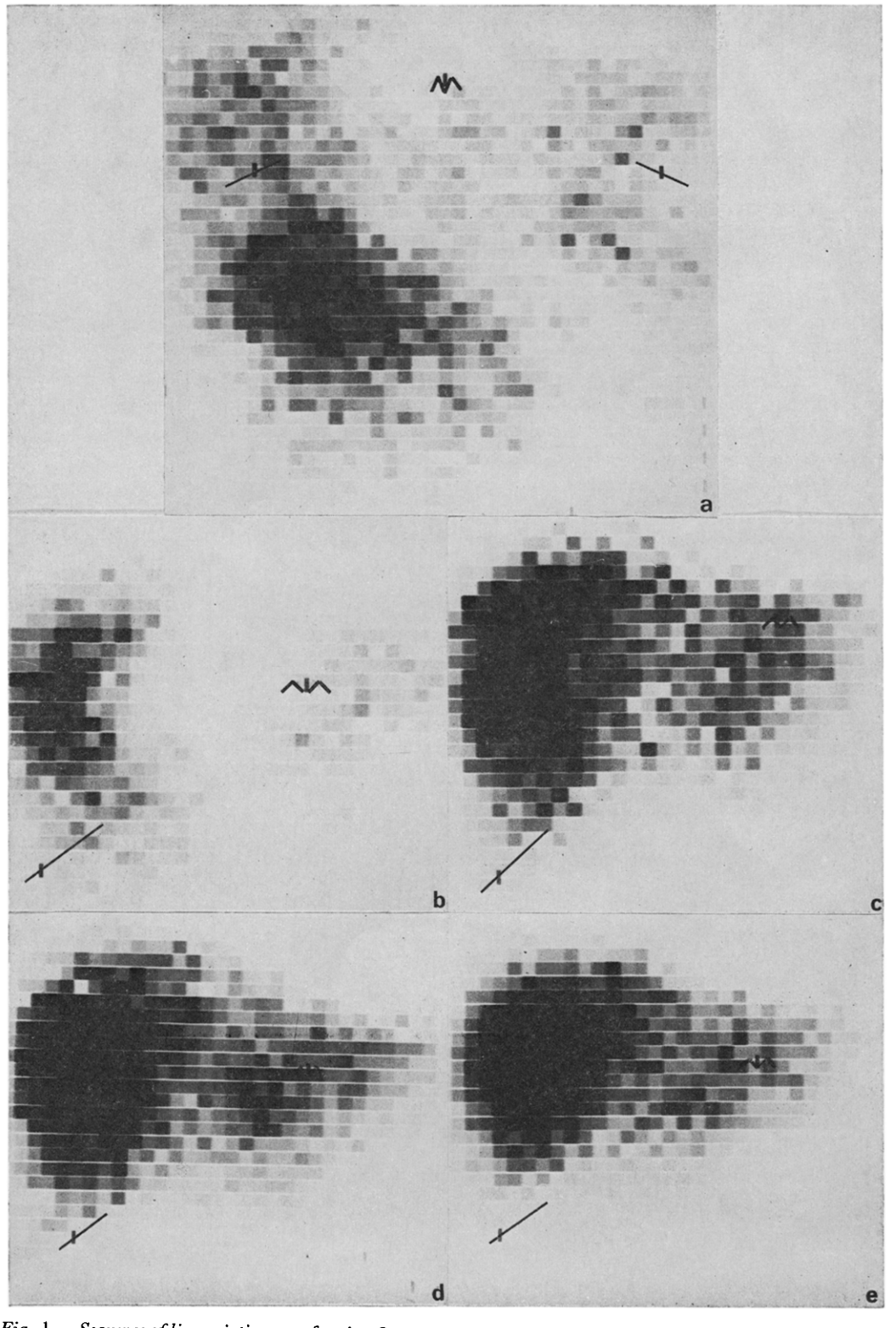

Fig. 1. *Sequence of liver scintigrams of patient* 3 *: picture* "a" *represents the scan made prior to treatment— pictures* "b" *to* "e" *are done* 3, 6, 9 *and* 14 *months after onset of treatment.*

peptidase were elevated in all cases. Serum transaminases were also abnormally elevated in all and total bilirubin in three. No patient had signs of severe liver failure.

Scintigraphy disclosed enlargement of the liver and multiple filling defects in all cases. Mean height of right lobe was 22·8 cm (21–27), (normal < 16 cm) and of left lobe 15 cm (12–18) (normal < 8 cm). Mean relative volume of neoplastic tissue was 51% (42–77%).

Evolution of liver involvement was estimated on consecutive scans. As illustrative of the evolution of all cases sequence of scans for patient 3 is shown in Fig. 1. Figure 2 represents the change in estimated relative neoplastic volume for all patients. It is expressed as per-

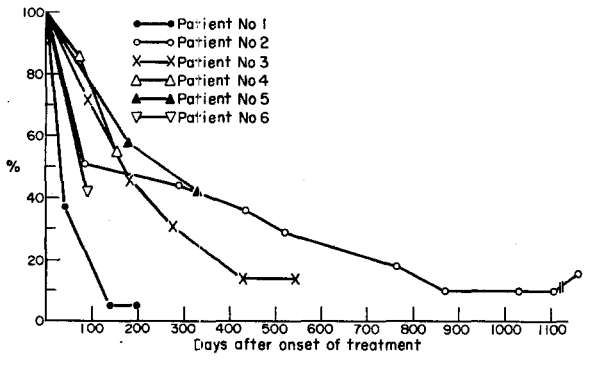

Fig. 2. Evolution of the relative volume of metastatic tissue in the liver, from onset of treatment, expressed as percentage of the initial value.

centage of the initial value. Already on the first control scan done between 40 and 182 days (average 94) after onset of therapy, the estimated relative neoplastic volume had dropped on the average to 58% (37–86%) of the initial value. At the time of the second control scan done between 141 and 382 days (average 219) (five patients) it had further dropped to 38% (5–55%). In patient 1, relative involvement dropped to 5% already after 140 days of treatment. In patients 2 and 3, relative neoplastic volume was reduced to lower than 15%; this value was maintained on subsequent scans for both patients. At the time of the last scan patient 2 had been off treatment for 22 months; six scans have been obtained since, the sixth shows an increase of the metastatic volume. Patient 3 was on treatment for 24 months and is now off treatment since 3 months. The remaining patients had a reduction of more than 50% of the neoplastic volume except for patient 4 who had a reduction of only 45%. Liver size decreased within normal limits both on liver scan and physical examination except in patient 4. Liver function tests came back to normal in all but one patient (patient 1) at time of the first control scan with the exception of alkaline phosphatase for which normalization occurred a few months later. Patient 1 had only a marked improvement of liver function tests. After a few months of treatment a control peritoneoscopy and needle liver biopsy could be performed in all patients except patients 1 and 5. There was no evidence of neoplastic disease neither on macroscopic visual inspection nor on microscopic examination. Patient 6 had a second control liver biopsy with still no evidence of neoplastic disease. Patient 1 died 3 months after treatment had been stopped (8 months after onset of treatment); no details are available concerning the cause of death. Patient 2, over 3 yr after onset of treatment and being off treatment for the last 22 months, relapsed with brain metastasis and hepatic reoccurrence (filling defect appeared on the scan and there was an increase to 15% of the relative metastatic volume). Patient 3 was treated for 2 yr and is off treatment for 3 months. Patient 4 died 2 months after cessation of treatment (9 months after onset of treatment); no details are available concerning the cause of death. Patient 5 is still on treatment for nearly over a year now. Patient 6 who had two control liver biopsies without evidence of neoplastic disease died of hepatitis (most probably of viral origin) two months after arrest of treatment (7·5 months after onset of treatment). The second biopsy was done a few weeks before death and showed signs of hepatitis and cirrhosis but no neoplastic cells.

DISCUSSION

This paper describes the cases of six patients with massive liver involvement from metastatic breast cancer. Involvement was convincingly documented by physical examination, liver function tests, liver scintigraphy and in five cases confirmed by laparoscopy or peritoneoscopy and positive liver biopsies. An original procedure of assessment using a stereological method indicated that the proportion of liver replaced by neoplastic tissue exceeded one third.

These patients were thus characterized by two factors of ominous therapeutic response as far as the liver is concerned, i.e. involvement of more than one third [2] and changes in the liver function tests due to metastatic disease [13]. Such patients are considered far too advanced to be likely to respond to endocrine treatments [1] and even to chemotherapy when single drug regimens are used [3]. Recent

reports [4–6, 13] suggest that the response rate of extensive liver metastases has considerably improved since the introduction of combination chemotherapy although assessment of response of liver metastases is admittedly difficult to carry out with accuracy [6, 7]. In our series of patients, the initial proportion of the relative liver volume occupied by metastatic tissue was ranging from 42 to 77%. This proportion dropped to between 3 and 24% after treatment. In five out of six cases, there was at least a 50% reduction. With regard to the remaining involvement it is difficult to differentiate between actual metastatic tissue and healing-related fibrosis. It is noteworthy that those patients who had a positive laparoscopy and liver biopsy prior to treatments became negative by these tests. These results were obtained with two different combination chemotherapies. Two patients received the original Cooper regimen. The remaining four were receiving a combination of an antioestrogen and cytotoxic drugs, which was recently reported as being one of the most effective chemotherapeutic regimens now available for breast cancer [11]. It remains to be established what proportion of patients with massive liver involvement will significantly benefit from these forms of treatment.

Besides illustrating the remarkable quality of response that can be obtained in these patients by combination chemotherapy both with regard to the extent of tumour regression and duration of remission, this paper presents a possible way of monitoring therapeutic response of metastatic liver. Clinical findings and liver function tests are part of the routine for the overall evaluation of metastatic involvement and evolution but are not completely reliable. It appears that liver scintigraphy brings about a suitable means of objective assessment of liver size and the method used of stereological estimation of relative metastatic tissue volume adds valuable information for monitoring therapeutic response. However, it should be stressed that laparoscopy and needle biopsy are required to ascertain the metastatic nature of the liver disease and particularly of its regression.

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Limitation of the Potentialities of Nephroblastoma Differentiation *In Vitro**

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Abstract—*The capacity of* 15 *human nephroblastomas to differentiate* in vitro *was studied. Chick and mouse embryonic tissues were used as inducers. Associating nephroblastomas with inducing tissues improved in* 6 *cases the growth and the survival of the explants and favoured in* 3 *cases a quantitative increase in the number of tubules when such structures were already present in the tumour* in situ. *No change was observed in* 9 *associated tumours and induction of tubules was not demonstrated with nephroblastomas which did not already contained such structures* in situ.

INTRODUCTION

An embryonic origin has often been suggested for nephroblastomas. A localized error in induction would allow an island of metanephrogenic mesenchymal cells to remain undifferentiated and dormant. The slow proliferation of this island could then progress unnoticed for several months or years to burst forth suddenly at a rate comparable to a normal growth of renal blastema [1] or with an accelerated and malignant evolution [2]. We therefore decided to study the *in vitro* potentialities of this tumour to differentiate or to continue to differentiate.

Our previous study on the behaviour of nephroblastoma in organ culture [3] has suggested the applicability of the *in vitro* association techniques between inductor and reactor tissues as first described by Grobstein [4] and currently used in studies of organogenesis [5]. These techniques have been applied to the study of a few tumours in man and in experimental animals [6–10] but only in a single case of a human embryonic tumour [6].

The differentiation of the metanephrogenic mesenchyme depends on ureteric induction in the chick embryo as was shown by Boyden [11] and by Grunwald [12]; Grobstein [13] later studied *in vitro* the epithelio-mesenchymal interactions required in the process of such differentiation of the mouse embryonic kidney. His collaborators later demonstrated that heterologous tissues could also induce the formation of tubules in metanephrogenic mesenchyme [14, 15].

Since human ureteric buds of 6–7 weeks-old embryos are not routinely available, we used chick ureteric bud [16] and mice mesencephalon [14] as inducer tissues. The validity of such an approach has been demonstrated by Waddington [17] concerning the zoological heterospecificity of induction and by Lustig *et al.* [9] concerning the application of such an induction to human tumours.

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MATERIAL AND METHODS

Control associations

Combinations between embryonic tissues were made on 32 series of 12 chick embryos (5½ days) by cultivating during one week the metanephrogenic mesenchyme either alone or in combination either with the homologous ureter or with embryonic chick or mouse mesencephalon. Dissociation of ureter from metanephrogenic mesenchyme were made mechanically and the absence of any remaining metanephric mesenchyme was carefully checked under dissecting microscope.

Associations with nephroblastoma

Fifteen Wilms' tumours of varying histology were used (Table 1). Tubular and pseudo glomerular differentiations were particularly

Table 1. *Nephroblastomas associated with embryonic inducers*

Tumours	Age	Sex	Differentiated elements			Combinations *in vitro*			Results of combinations
			tubular	preglom	muscul.	(ch. ur)	(ch. Mes)	(M.Mes)	
1 (967)	14 months	♀	+	+	+	−	+	−	no change
2 (970)	14 months	♀	+	−	+	−	+	−	no change
3 (1121)	2 years	♂	micro cysts	−	−	+	−	−	longer survival
4 (1159)	6 years	♂	−	−	−	+	−	−	no change
5 (1163)	18 months	♀	+	+	+	+	+	−	(necrotic)
6 (1178)	9 years	♂	+	−	−	+	+	−	longer survival
7 (1196)	6 years	♀	+	+	+	+	+	+	no change
8 (1209)	18 months	♂	+	+	+	+	−	+	no change
9 (1215)	5 years	♂	+	+	−	+	−	+	more tubules
10 (1221)	7 years	♀	+	+	−	+	−	+	more tubules
11 (1224)	17 months	♂	+	−	−	−	−	+	no change
12 (1237)	4 months	♂	−	−	+	−	+	+	no change
13 (1252)	2 years	♀	−	−	−	−	−	+	no change
14 (1256)	4 years	♀	+	+	−	−	−	+	longer survival
15 (1320)	1 year	♀	+	+	+	−	−	+	more tubules

ch = chick, M = mouse, ur = ureter, Mes = mesencephalon

clear in eight of them. Three nephroblastomas, including one irradiated metastasis, showed no tubular structures. Five of the patients had been treated preoperatively with chemotherapy or irradiation. The patients' age varied from 4 months to 9 years.

The tumours were grown as organ cultures using "Falcon" plastic organ culture dishes and disks of "Millipore" filter supports [3]. The cultures were grown at 37°C in Eagle's medium (Institut Pasteur, Paris) which was supplemented with 10% decomplemented calf serum and antibiotics (penicillin 200 u/ml, streptomycin 0·1 mg/ml) in the presence of air containing 5% CO_2.

The inducer tissues (the ureter or the mesencephalon taken from 3½ or 5½ day chick embryos or the mesencephalon of 11–15 day-mouse embryos) were combined with human tumour fragments *in vitro* (Table 1). The associations of embryonic tissue with tumour were made during the first two weeks of the organ culture of the tumour and were cultivated together for an additional 8 days. This association of the two tissues was carried out by placing directly adjacent to each tumour explant an identical volume of inductor tissue freshly removed from an embryo.

For each nephroblastoma, a minimum of 12 tumour explants were brought into contact with the inductive tissue. After termination of the experiments the explants were fixed in alcoholic Bouin solution, embedded in paraffin and 5μm sections were cut and stained with hematoxylin-eosin or with periodic acid–Schiff.

RESULTS

Control associations (*Table* 2)

Cultivated alone for one week the chick metanephrogenic mesenchyme condensed (Figs. 1 and 2) but did not form real tubular structures. If the metanephrogenic mesenchyme was cultivated together with ureter for one week it underwent differentiation and formed tubules (Fig. 3). Similar results were obtained when ureter and metanephrogenic mesenchyme were removed and cultured as one block. The appearance of these tubules was variable. Tubules of secretory type, edified from the mesenchyme, are seen close to rather large ureteric tubules. Secretory type of tubules are small and dark, showing sometimes a well differentiated S shaped tubule. A few preglomerular formations were also observed. When the chick metanephrogenic mesenchyme was associated with either homologous mesencephalon or with the mesencephalon of 11- to 15-day-mice embryos, it was found that the chick mesencephalic tissue had an inductive capacity comparable to that of homologous ureter, while mouse-chick associations were less powerful in inducing renal differentiations. The results are summarized in Table 2.

Associations with nephroblastoma

The survival of tumour fragments in organ culture was similar to what we have pre-

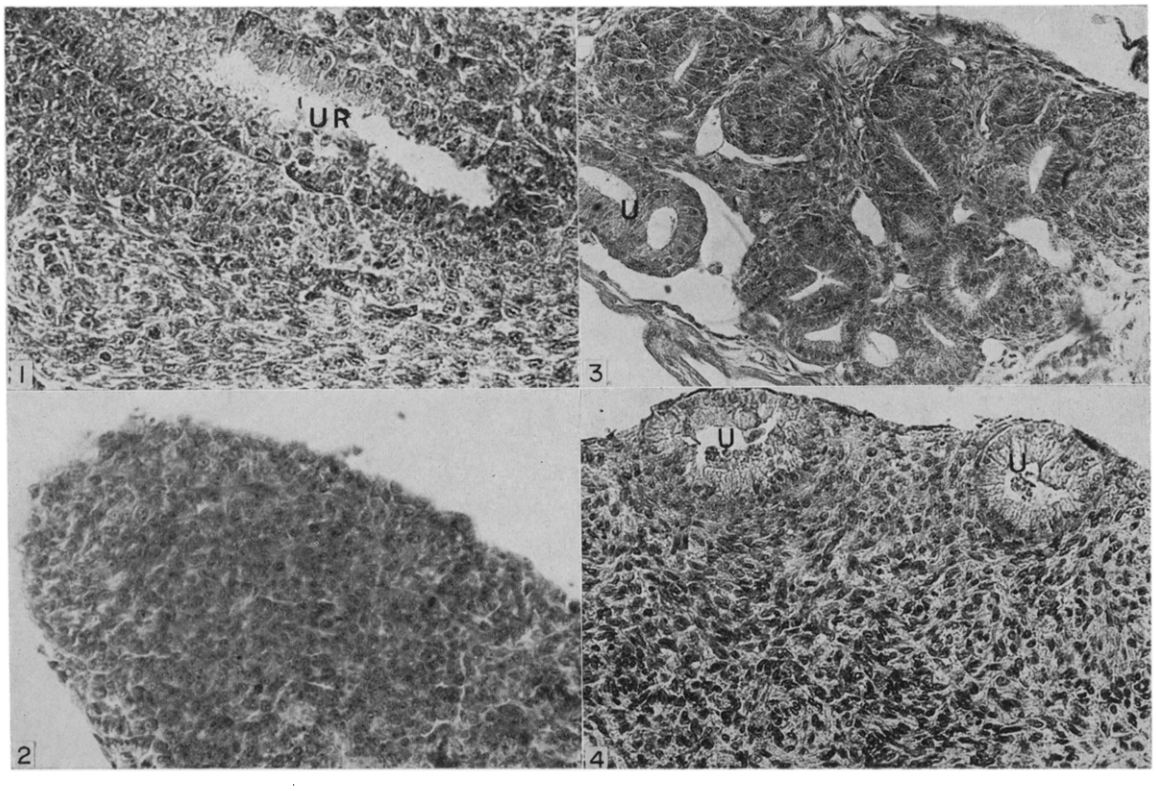

Fig. 1. *Metanephrogenic mesenchyme and ureteric bud (UR) of a* $5\frac{1}{2}$ *day chick embryo. Note the cellular condensation of mesenchyme near the ureter* (*HE* × 400).

Fig. 2. *Metanephrogenic mesenchyme of a* $5\frac{1}{2}$ *day chick embryo cultivated alone for one week. Note the cellular condensation of the whole explant* (*HE* × 400).

Fig. 3. *Reassociation of metanephrogenic mesenchyme and ureter of a* $5\frac{1}{2}$ *day chick embryo after* 8 *days in culture. The cells of the ureter (U) have proliferated but remain well defined. There are tubules of varying maturity* (*HE* × 250)

Fig. 4. *Nephroblastoma (No.* 1121) *after* 24 *days* in vitro *and in contact with chick ureter (U) for* 6 *days. No tubule formation was induced. Note the good intermixing of the cells* (*HE* × 250).

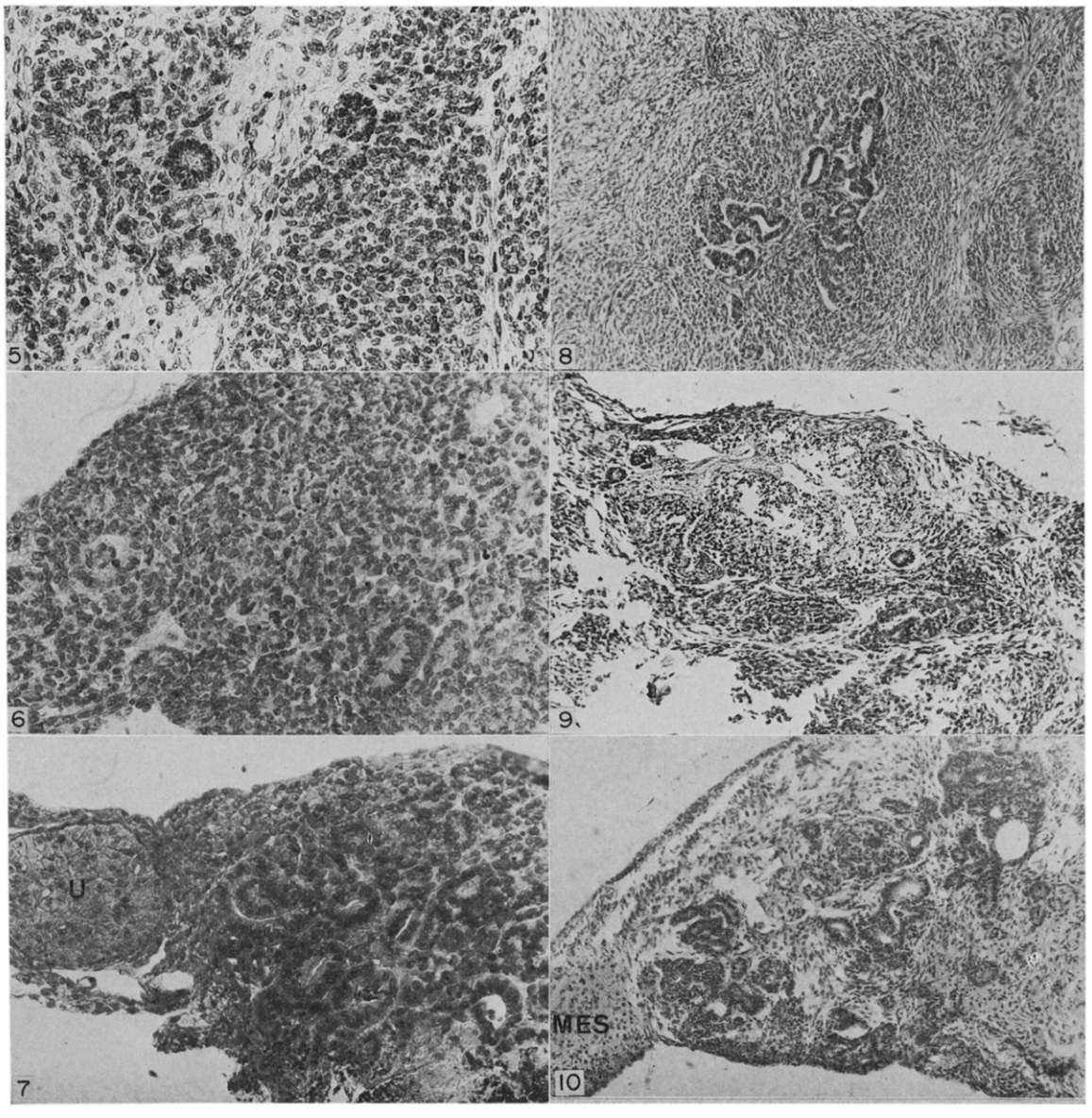

Fig. 5. *Nephroblastoma* (*No.* 1215)*: Histology before culture. There is tubular differentiation* (*HE* × 250).

Fig. 6. *Nephroblastoma* (*No.* 1215) *after* 9 *days in culture. The tubular formations persist and preglomerular structures are also present but all are dispersed* (*HE* × 250).

Fig. 7. *Nephroblastoma* (*No.* 1215) *grown with the ureter* (*U*) *of a* 5½ *day chick embryo. There is a good inter-mixing of the cells. The tubular and preglomerular formations are more numerous and more regrouped than in the control explants but are no more mature than in the same tumour before culture* (*see Fig.* 5) (*HE* × 250).

Fig. 8. *Nephroblastoma* (*No.* 1320). *Histology before culture showing tubular formation and preglomerular structures* (*HE* × 100).

Fig. 9. *Nephroblastoma* (*No.* 1320) *after* 9 *days in culture. Few differentiated structures were present in the half-necrosed explant* (*HE* × 100).

Fig. 10. *Nephroblastoma* (*No.* 1320) *after* 9 *days in culture and grown in contact with* 11 *day mouse embryo mesencephalon* (*MES*) *for* 7 *days. The tubular formations are more numerous than in the control explants* (*HE* × 100).

Table 2. Control associations between normal embryonic tissues: Results after 7 days in vitro

Cultures / *In vitro* differentiation	Chick				Chick metan. mesench. + mice mesenceph.
	Metan. mesench.	Complete metanephros	Metan. mesench. + ureter	Metan. mesench. + mesenceph.	
+	0	15	15	10	4
±	2	5	5	1	5
−	30	1	0	2	5
Total No. of cultures	32	21	20	13	14

\+ Tubular and preglomerular differentiation.
± Reorganized metanephrogenic mesenchyme with rare primitive tubules.
− No differentiation.

Table 3. Mean number of tubules per nephroblastoma explant as combined or not with embryonic inducer tissues

No.	Duration of the culture	Duration of the combination	Nephroblastoma alone	Nephroblastoma + chick ureter	Nephrobl. + mouse mesenceph.
9 (1215)	5 days	5 days	8·8	12	12
	9 days	9 days	12·4	22	18·3
	12 days	4 days	15·2	18·2	17·3
	16 days	7 days	14·2	26·6	17
10 (1221)	17 days	7 days	16·8	21	28
15 (1320)	6 days	4 days	5	no done	11
	9 days	7 days	6·7	no done	13

viously observed [3] and is generally reduced by preoperative treatment of the patient or the presence of muscular elements in the explants. No new tubule formation was induced by any of the combinations used with the 3 nephroblastomas which did not contain tubules *in situ*. In the nephroblastomas which originally contained tubules no further qualitative maturation became evident. Nevertheless, a quantitative increase of the tubules was observed in 3 cases.

Only one nephroblastoma (# 5) gave necrotic results in culture whether combined or not with inducer tissues. This result may be explained by the presence of muscular components in the tumour and by the preoperative treatment of the patient.

The combinations of tumour explants with chick embryo ureter led to a good intermixing of the cells. Eight nephroblastomas were combined (Table 1). One tumour was necrotic before combination (# 5). Three tumours, two differentiated (# 7, 8) and one not (# 4, Fig. 4) seemed unaffected by the association procedure since no difference was noted with the control explants. In the four other tumours we noticed a better survival of the explants when associated with chick embryo ureters. The size of combined explants increased or was maintained with culture time; the cellular aspect looked more "healthy" (nucleoli of normal size, good delimitation of the cells, mitoses) than in the control explants. In two of these nephroblastomas (# 9, 10) the number of tubules was also increased as compared with the *in vitro* isolated tumour (Table 3 and Figs. 5–7). In the third case (# 3) tubules were absent and in the fourth (# 6) only rosettes were present.

The nephroblastoma explants associated with chick or mouse mesencephalon remained in contact with each other but less completely than when associated with ureteric bud. Only 1 out of 6 combinations of the tumour explants with chick mesencephalon showed a survival of the culture which was comparable to the tumour–ureter combinations (# 6).

Nine tumours were associated with mouse embryo mesencephalon (Table 1). In 5 cases, two well differentiated (# 7, 8) and three not (# 11, 12, 13), no change occurred in the combined explants as compared with the

control explants. In the four other cases a longer survival was noted in the combined explants. In three such cases (# 9, 10, 15) associated nephroblastoma explants contained more tubules than the growing alone controls (Table 3 and Figs. 8–10). In the fourth case tubules were not numerous enough to be significantly evaluated in the explants.

With three differentiated nephroblastomas (# 9, 10, 15) the number of tubules per explant was calculated in relation with experimental conditions. From these measures the mean number of tubules per nephroblastoma explant was established and reported on Table 3 in relation with the association procedure and the duration of the culture. A two factor variance analysis was performed (# 9) = it showed that the factors association and time played a role (5% signification in both cases). A comparison of the mean number of tubules of the three groups (nephroblastoma alone, nephroblastoma + chick ureter, nephroblastoma + mouse mesencephalon showed that

(1) combination with ureter differs significantly from controls ($P = 0{\cdot}02$)
(2) combination with mouse mesencephalon differs at the limit of significance ($0{\cdot}10 > P > 0{\cdot}05$) for case 9 but was significant for case 10 ($P = 0{\cdot}05$).

With regard to the time factor the results during the first week *in vitro* differed from longer culture periods due to the fact that nine day cultures showed the higher relative incidence of tubules.

DISCUSSION

The purpose of our experiments was to test the possibility of nephroblastoma cells either to differentiate or to continue a differentiation which was already underway.

In the control experiments we tested the tubule inducing capability of several kinds of embryonic inducers some of which were heterospecific. Furthermore, we showed that chick mesencephalon can induce chick metanephrogenic mesenchyme to differentiate. We also demonstrated that heterospecific induction of renal differentiation is possible while using the combination of chick metanephrogenic mesenchyme with mouse mesencephalon. These latter two combinations were not previously tried by others. The inductive capability of chick ureter and mouse mesencephalon on homospecific metanephrogenic mesenchyme has already been demonstrated *in vitro* [14, 16]. Lombard and Grobstein [14] were also able to demonstrate the inducing capacity of 9 day chick embryonic mesencephalon on mouse metanephrogenic mesenchyme.

In our studies of nephroblastomas, we observed that they retained their general characteristics in organ culture. The association of tumour fragments with various embryonic tissues improved in some cases (6 out of 15) the *in vitro* survival of the fragments and maintained differentiation within them. Such *in vitro* stimulation of tumoral growth has been previously obtained by combining some embryonic tissues with tumours [18]. It is believed that embryonic tissues and yeast extracts [19] bring trophic factors to the culture, thus prolonging survival of the tumour cells. Biochemical analysis has also been attempted to demonstrate the nature of such stimulation [20]. Such studies suggest a metabolic cooperation (by enzyme product exchange) between the tumour and the embryonic cells as it has been observed in certain mixed cultures of mutant and non mutant cells [21].

Our results show an increase in the number of tubules in 3 nephroblastomas associated with embryonic tissues. This increase may be related to the nutrient action of embryonic tissues. A specific stimulation of tubule elongation produced by the inducer tissue during the second step of renal tubulogenesis [22] is an alternative possibility. Ellison *et al.* [8] have observed renal differentiation in 1 of 28 transplantable tumours which originated spontaneously in rat kidney when they associated rat or mouse embryonic tissues *in vitro* with tumour explants. This tumour originally had a few tubular formations *in situ* and differentiation appeared while the number of tubules in the associated tumour explants increased as the duration of culturing increased. The authors concluded that these tissue interactions showed no inductive capacity but were enhancing the latent ability of the renal tumour to differentiate.

Successful *in vitro* induction of another type of embryonic renal neoplasm have been reported. This tumour, a congenital mesoblastic nephroma, was often identified as congenital Wilms' tumour before it was described as a separate entity by Bolande *et al.* [23]. Crocker and Vernier [6] described *in vitro* induction of renal structures in such a tumour using 11-day-old fetal mouse dorsal brain or spinal chord as inducers. The renal differentiation progressed for 96 hr and produced "typical s-shaped fetal nephrons". This capacity to differentiate would appear to be correlated with the embryonic origin of the congenital nephroma and may be

one of the reasons for its benign clinical behaviour. Bolande [24] did not agree with the conclusions drawn by Crocker and Vernier because, in his experience, that type of tumour contains a natural mixture of normal and dysplasic nephronal elements. We had the opportunity to make *in vitro* inductive associations of two mesoblastic nephroma with mouse embryonic mesencephalon. Tubular structures were present in both tumours *in situ* as well as in the explants, whether combined with the inducer or cultured alone. However, the *in vitro* survival of the explants was not prolonged enough to permit a final conclusion on this matter.

The nephroblastomas which we studied responded only partially to the inducer tissues and for the most part retained without any change their more or less blastematous appearance. Induction of tubules was not demonstrated with the nephroblastomas which did not already contain such structures *in situ*. The reason for this may be that the embryonic inducers we used were not adequate, and further experiments using human embryonic inducers are certainly needed. The availability of such material is, unfortunately, very limited. Nevertheless, the numerous examples of heterospecific renal induction indicate that species differences are probably not the major obstacle to morphogenetic stimuli in our culture situation.

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Carcinoembryonic Antigen (CEA) in Patients with Breast Cancer*

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Abstract—*Plasma CEA concentrations were measured in normal women and in patients with both primary and advanced breast carcinoma by means of a radioimmunoassay utilising rabbit anti-CEA antiserum. Fifty-five per cent of women with primary breast cancer had detectable levels of CEA (>0.1 U/ml), as compared to 13% in a random selection of the female population. The incidence of detectable CEA levels in benign conditions of the breast was less than 10%, but the largest incidence of detectable levels of CEA (70%) was in the group of patients with advanced breast carcinoma.*

In patients with primary cancer, no correlation was observed between the plasma CEA levels and either the presence of oestradiol-17β receptor proteins in the tumours or the menstrual status of the patient.

The effect of anti-oestrogen therapy on plasma CEA concentration in patients with advanced breast cancer was studied. A greater proportion of patients classified as clinical non-responders showed increased plasma CEA concentrations and fewer showed decreased CEA levels than in the case of the patients who responded to treatment.

INTRODUCTION

SINCE the first demonstration of the presence of carcinoembryonic antigen (CEA) in neoplastic tissue [1], elevated plasma levels of CEA have been observed in patients with tumours at various sites [2–5]. There has been considerable interest in using plasma CEA level measurements as an *in vitro* cancer test, but it has been shown that such measurements in apparently healthy individuals are not a suitable method for screening a population because negative results may be obtained in subjects with early carcinomata, whilst some non-malignant disorders are associated with elevated CEA levels [2, 4].

The value of plasma CEA measurement is more apparent in the management of patients whose diagnosis has already been established. It has been reported [2] that metastatic spread of breast cancer correlates with plasma CEA levels and that women with detectable CEA levels (> 2·5 ng/ml) had a faster recurrence rate after mastectomy than patients with lower CEA titres [6].

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The present study was designed to determine the significance of plasma CEA levels in patients with advanced and primary breast cancer and in predicting the response to therapy with the anti-oestrogens clomiphene and tamoxifen which have been utilised in the management of breast cancer [7, 8].

MATERIAL AND METHODS

1. *Subjects*

The normal women in this study were volunteers resident in South Wales and were totally unselected women from the general population. We are grateful to Dr. P. C. Elwood of the M.R.C. Epidemiology Unit (South Wales) for providing these plasma samples.

The patients with primary breast cancer consisted of 74 women from whom blood was taken at the time of mastectomy.

The advanced breast cancer group comprised 95 patients with either local or widespread recurrence of the disease. In the groups treated with anti-oestrogens, samples were collected before the commencement of the therapy and at 3 months afterwards. Patients were given either tamoxifen (ICI 46474)

20 mg twice a day or clomiphene 100 mg orally twice a day for a minimum of one month. The clinical response was assessed as described by Golder *et al.* [9].

The plasma samples from the bronchial carcinoma patients were provided by Dr. J. V. Pritchard of Velindre Hospital, Cardiff.

The concentration of oestradiol-17β receptor proteins in the tumours was determined by the method of Powell-Jones *et al.* [10].

Blood was taken into lithium-sequestrene tubes and, after centrifugation, the plasma was stored at −20°C. Aliquots of plasma were transported frozen to the Tenovus Institute.

2. *CEA, ^{125}I-CEA, and anti-CEA antiserum*

CEA was a generous gift from Dr. D. J. R. Laurence of the Chester Beatty Institute, London. CEA was iodinated with $^{125}I^-$ by the method of Greenwood *et al.* [11]; the specific activity ranged from 80–120 μCi/μg.

Anti-CEA antiserum was raised in New Zealand white rabbits by intradermal injection [12] and was used throughout at a dilution of 1:4800 with normal rabbit serum (NRS) diluted 1:300. At this dilution, 30% of the radioactively labelled CEA was precipitated in the radioimmunoassay.

3. *Radioimmunoassay for CEA*

All dilutions were made in PBSE (0·05 M phosphate pH 7·4 containing 0·9% (w/v) NaCl and 1 mM EDTA). 100 μl (about 10^4 cpm) of ^{125}I-labelled CEA was mixed with 200 μl of either pooled plasma, pooled plasma containing a known amount of CEA, or the unknown sample. The plasma samples used to constitute the pool contained no detectable CEA. 200 μl Anti-CEA antiserum diluted 1:4800 in normal rabbit serum (NRS) 1:300 was added and the mixture incubated for 24 hr at 4°. 200 μl of sheep anti-rabbit antiserum diluted 1:30 was then added and the mixture incubated for a further 24 hr at 4°. The precipitate was separated by centrifugation in a Mistral 4L for 30 min at 800 *g* at 4° and counted in a Nuclear Chicago Gamma Counter.

For each level of non-labelled CEA and each plasma sample, incubations were carried out in duplicate. The results are expressed in units (U) of the First British Standard for Carcinoembryonic Antigen (CEA) C73/601 [13]. A mean value of 24 ng of the local standard of CEA was found to be equivalent to 1 U of the British Standard CEA.

4. *Analysis of RIA data*

A composite standard curve is shown in Fig. 1, together with values for the Coefficient of Variation of the estimations. The values of the response metameter B/B_0 were fitted to the CEA concentration using a 4-parameter model [14]. From the standard curve the maximum sensitivity of the assay, defined according to Kaiser and Specker [15], was found to be 0·1 U/ml and the probability of detection of

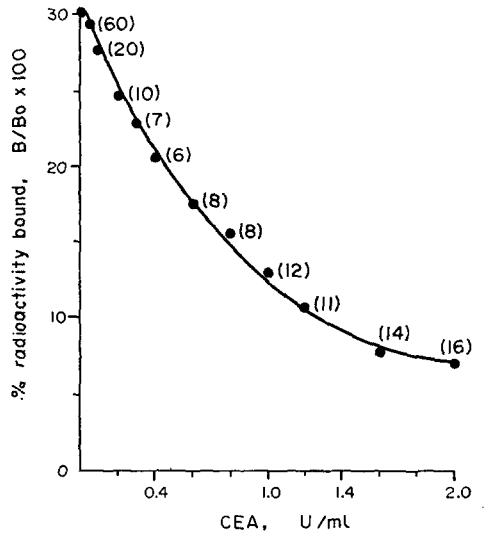

Fig. 1. Composition of standard curves prepared with anti-CEA antiserum from 6 successive assays. The coefficient of variation of the determination at each point on the curve is indicated.

this quantity from zero for the duplicate estimates used in this analysis was approximately 84%. The actual assay sensitivity for specified ranges was estimated by the method of Snedecor [16]; the results are shown in Table 1.

Table 1. Precision of plasma CEA determination expressed as estimates of the standard deviation (s) of results from the means

Range of CEA concentrations (U/ml)	*N*	s	Sensitivity ($P = 0·01$)
0 –0·4	46	0·03	±0·06
0·41–0·8	18	0·07	±0·13
0·81–1·6	10	0·18	±0·33

Pre- and post-treatment CEA levels of patients in the tamoxifen/clomiphene trial were compared using Student's *t* test. Values were considered different if $P < 0·05$. Patients whose pre-treatment CEA levels were below the

limit of sensitivity of the assay were excluded from this analysis.

RESULTS

Plasma CEA levels in normal women and patients with primary breast cancer

The levels of plasma CEA for an unselected group of women in a normal population and for patients with a variety of malignant diseases are shown in Table 2. The results show that detectable levels of CEA occurred in about 13–14% of a random selection of the female population when no account had been taken of the state of health of the volunteers.

In patients diagnosed as having primary breast carcinoma, the incidence of elevated CEA levels was about 54%, while less than 10% of a small group with benign breast disease had detectable CEA titres. However, in patients with advanced breast cancer, there was a marked increase in the number with elevated CEA levels, about 70% having levels greater than 0·1 U/ml and about 40% of these being greater than 0·4 U/ml. In comparison, a non-steroid-dependent tumour, bronchial carcinoma, showed detectable levels in about 45% of cases.

The relationship between plasma CEA levels in patients with primary breast cancer and the presence of oestradiol receptors in these tumours is shown in Table 3a. Patients with an oestradiol receptor concentration of less than 5 fmol/mg protein were defined as being receptor negative while those with a value greater than 5 fmol/mg protein were classified as receptor positive. In both receptor positive and receptor negative tumour bearing patients, the plasma CEA levels were elevated in 50–55% of cases.

A similar percentage of elevated plasma CEA levels was observed when the primary breast cancer patients were classified according to their menstrual status (Table 3b).

Plasma CEA levels in patients with advanced breast cancer and response to treatment with anti-oestrogens

The pretreatment plasma CEA levels in a group of 21 women with advanced breast cancer who were subsequently treated with tamoxifen

Table 2. Plasma CEA levels in the normal population and in various disorders

		Plasma CEA (U/ml)		
Condition	*N*	<0·1 (not detectable)	0·1–0·4	>0·4
Normal women	200	173 (86·5%)	27 (13·5%)	0
Primary breast cancer	74	33 (44·8%)	38 (51·3%)	3 (3·9%)
Advanced breast cancer	95	31 (32·6%)	36 (37·9%)	28 (29·5%)
Benign breast disease	22	20 (90·9%)	2 (9·1%)	0
Bronchial cancer	49	27 (55·1%)	15 (30·6%)	7 (14·3%)

Table 3. Comparison between plasma CEA levels, tumour oestradiol-receptor concentrations, and menstrual status in patients with primary breast cancer

a. Plasma CEA levels and oestradiol-receptor concentrations.

Plasma CEA (U/ml)	E_2 receptor +ve	E_2 receptor −ve
<0·1	14 (48·3%)	14 (45·1%)
0·1–0·4	14 (48·3%)	16 (51·6%)
>0·4	1 (3·4%)	1 (3·3%)
	N = 29	31

b. Plasma CEA levels and menstrual status

Plasma CEA (U/ml)	Pre-menopausal	Post-menopausal
<0·1	9 (50·0%)	19 (48·7%)
0·1–0·4	9 (50·0%)	17 (43·6%)
>0·4	0	3 (7·7%)
	N = 18	39

are shown in Table 4a, which also shows the levels in a group of 30 women subsequently treated with clomiphene. In the case of the tamoxifen series, elevated plasma CEA levels were present in about 60–70% of responders and non-responders alike, while this proportion was even higher in both classifications of the clomiphene series.

The effects of treatment with anti-oestrogens on plasma CEA levels are shown in Table 4b. Those patients whose pre-treatment plasma CEA levels were below the limit of sensitivity of the assay were excluded from this analysis; no positive pre-treatment value fell below the limit of sensitivity following treatment. Three months after the commencement of treatment, 19 patients were classified as responding to either therapy. Of these, 37% (7/19) showed no alteration in plasma CEA levels, while in 6/19 the level of CEA decreased in response to treatment. However, in 6 cases there was a significant increase in plasma CEA levels after 3 months.

Of the 22 patients classified as non-responders to either treatment 50% showed no changes in CEA levels after treatment. However the CEA titre fell in only one patient, and rose significantly in 10/22.

When the results were expressed as percentages of the initial CEA level (Fig. 2), there was a significant difference between the responders and non-responders. The mean and standard error for the responders was $101{\cdot}1 \pm 13{\cdot}5$ compared to $143{\cdot}6 \pm 13{\cdot}6$ for the non-responders.

DISCUSSION

In this study elevated plasma CEA levels have been demonstrated to occur in about 13% of a totally unselected number of the female population. This finding is in agreement with two previous studies [17, 18] in which elevated CEA levels in plasma were found in 11–16% of the population. In contrast to this, however, some studies [2, 19] have shown no positive CEA levels, while that of Wang *et al.* [6] found

Table 4. Pre-treatment plasma CEA levels in advanced breast cancer and the clinical response to anti-oestrogen therapy

a. Pre-treatment CEA levels

Patients	Treatment	Plasma CEA (U/ml) <0·1	0·1–0·4	0·4–1·0	>1·0
Responders ($N = 23$)	Tamoxifen	4	4	2	0
	Clomiphene	0	9	1	3
	Total	4	13	3	3
Non-responders ($N = 28$)	Tamoxifen	3	6	2	0
	Clomiphene	1	9	5	2
	Total	4	15	7	2

b. Response to anti-oestrogen therapy after 3 months

Patients	Treatment	Serial CEA levels Increase	No change	Decrease
Responders $N = 19$)	Tamoxifen	2	2	2
	Clomiphene	4	5	4
	Total	6	7	6
Non-responders ($N = 22$)	Tamoxifen	4	4	0
	Clomiphene*	6	7	1
	Total	10	11	1

*Two non-responders to clomiphene were not sampled at 3 months.

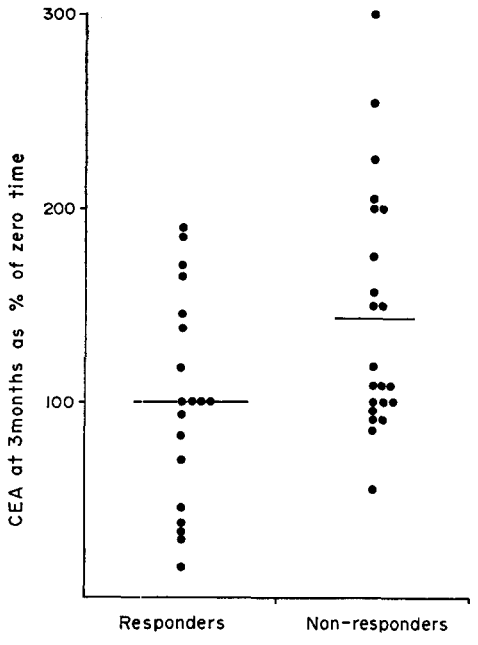

Fig. 2. Effect of anti-oestrogens on plasma CEA levels. Plasma CEA concentrations at 3 months are plotted as a percentage of pre-treatment values. Mean ± S.E.M. for responders was 101·1 ± 13·5 *and for non-responders was* 143·6 ± 13·6.

approximately 35% of a random group of women with raised levels of CEA.

The percentage of patients with primary breast carcinoma with an increase in plasma CEA concentrations was about 55%, a figure which is higher than the previously recorded values of 33–46% [2, 6]. However, the CEA levels tended to be lower than those from patients with advanced breast cancer. The incidence of high CEA levels in benign conditions of the breast was low (< 10%) in agreement with the findings of Laurence *et al.* [2]. None of these samples had levels in excess of 0·4 U/ml.

The comparison between plasma CEA levels and the presence or absence of oestradiol-17β receptor proteins in the tumour (Table 2) shows that in both the hormone-dependent and hormone-independent tumours, elevated CEA levels occur in approximately 50% of cases. Similarly, with the menstrual status of the patient, about 50% of both pre- and post-menopausal women had increased levels of CEA. Thus it is apparent that there is no correlation between the plasma CEA level and the hormonal status of the patient.

A much larger proportion of elevated plasma CEA levels can be seen in patients with advanced or metastatic breast carcinoma. In this series, 68% of patients had positive CEA levels and about 30% had levels in excess of 0·4 U/ml. These values are comparable to those from previous studies where 60–80% of patients with advanced breast cancer had increased plasma CEA levels [5, 17, 18].

The most valuable aspect of CEA measurement at present appears to be in the monitoring of therapy. In this study we have investigated the effect of anti-oestrogen therapy on plasma CEA levels in patients with advanced breast cancer. Although there was no correlation between pre-treatment levels of CEA and clinical responsiveness, there were marked differences between the groups assessed as responding or not responding to treatment when changes in CEA levels over the 3-month treatment period were considered. A significant fall in plasma CEA was observed in only one clinical non-responder, but in 6/19 responders. Although raised CEA levels were observed in patients in both groups, the percentage of patients with increased values was higher among the non-responders. Similar results have been obtained for breast cancer patients in response to chemotherapy [20]. Figure 2 shows that a partial separation of the populations of responders and non-responders is possible on the basis of the data presented here, but more detailed studies are clearly required before the value of serial determination of CEA as a means of assessing tumour regression can be assessed.

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Effect of BCG Treatment on the Evolution of Cell-Mediated Immunity in Mice Bearing Transplantable Syngeneic Tumors*

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Abstract—*The effect of BCG administration on the evolution of* in vitro *cell-mediated immunity in relation to tumor growth was studied in C3HeB/Fe mice carrying transplantable, syngeneic mammary TM*1 *tumors. Intradermal (i.d.) injection of BCG, 7 days before inoculation of tumor cells, inhibited significantly the tumor growth, whereas similar injection of BCG, 7 days after tumor graft, when the mice had already a palpable nodule, showed no effect. These results were correlated to a great extent with those obtained from* in vitro *colony inhibition (CI) tests using peritoneal cells (PC) of the same mice: from the beginning of the tumor growth, the CI activity of PC from mice which received BCG before tumor graft was significantly higher than those of the other groups of mice and this elevated activity was still high at the* 35*th day after tumor graft, when the PC from control non-treated mice or from mice which received BCG after tumor graft were entirely inactive. When BCG was inoculated directly into the growing tumors, significant retardation of the tumor growth was observed. This tumor inhibiting effect following intratumoral (i.t.) inoculation of BCG was much more pronounced in mice that had been presensitized with BCG before tumor graft. The* in vitro *PC-mediated immunity of these mice showed concurrently a significant increase at* 2 *weeks after i.t. inoculation of BCG, while that of the control non-treated mice was still at the lowered level of activity. Intradermal injection of BCG* 7 *days before tumor operation accelerated significantly the post-operative recovery of PC-mediated immunity, whereas similar inoculation of BCG* 3 *days after tumor operation resulted a rather adverse effect.*

INTRODUCTION

SINCE the demonstration that BCG was a potent non-specific immunostimulant enhancing host resistance against a variety of animal tumors [1, 2], numerous attempts have been made to interfere, using this microorganism, with the growth of experimental tumors in different animal species [3–5] as well as in clinical malignancies [6, 7]. Encouraging results were reported along this line, but still, optimal conditions in which the most beneficial effect of tumor inhibition can be obtained in relation to the mechanisms governing the action of the BCG are not well established. Moreover, confusingly enhancing effects on tumor growth after BCG administration in certain situations have been described [8–10].

In a more general way, it can be assumed that regression or enhancement of growth of a tumor which possesses tumor specific antigens will result from a balance between specific cell-mediated immune response acting mainly in favor of tumor rejection, and serum blocking factors, supposedly weakening or suppressing this reaction [11]. Procedures increasing cell-mediated immunity and, eventually, lowering or eliminating the action of the blocking factors might be an essential clue for successful tumor

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inhibition according to this concept. The precise role possibly played by BCG in this complex system of antitumor immune reactivity has still to be explored.

We have previously reported several series of experiments concerning a characteristic pattern of evolution of specific cell-mediated antitumor immunity studied by *in vitro* tests in several tumor-host systems [12–18]. We could demonstrate in this way that cell-mediated immunity was evident at the onset of tumor growth, but disappeared when tumors grew to a larger size, the animal entering an immunological "eclipse" state. This immunity, however, reappeared after a delay of 2 to 3 weeks following tumor removal.

The purpose of the present study was, on the basis of previously established trends of "natural" evolution of cell-mediated tumor-specific immunity, to check the additional effect of BCG treatment.

MATERIAL AND METHODS

Mice

One-month-old female inbred C3HeB/Fe mice were used. They were obtained from the Animal Selection Center, Orléans, France. Five to 7 mice were caged and supplied with food pellets and water *ad libitum*.

Tumor-cell line

A transplantable tumor-cell line, TM1, originally developed from a spontaneous mammary tumor of a C3H mouse, was used [14]. It was routinely cultivated in Eagle's minimum essential medium (MEM) supplemented with 10% non-inactivated fetal calf serum, 100 I.U. of penicillin and 100 μg of streptomycin per ml. Monodispersed cell suspensions were prepared by treatment of cultured cell monolayers with 0·05% trypsin-EDTA solution and used for production of tumors in mice or as a source of tumor target cells for *in vitro* cytotoxic tests. Tumor-specific antigenicity of these cells was proved *in vivo* by rejection of tumor cell challenge inoculation by syngeneic mice which had been actively immunized with irradiated tumor cells.

Bacillus Calmette-Guérin (BCG)

BCG Pasteur stain was obtained from the Pasteur Institute, Paris, as freeze-dried vaccine. The microorganisms were routinely cultivated on Jensen's solid medium from which serial subcultures were made in Dubos liquid medium to obtain homogenous BCG suspensions. After 3 washings with saline by centrifugation, final BCG suspensions were prepared in saline for animal inoculations. The wet weights of the bacteria were determined by comparing their densities with those of standard BCG opacimeter scale (Institut Pasteur, lot No. 179 bis, 5×10^6 viable units/mg) with the aid of a spectrophotometer.

In vitro *cytotoxicity tests*

Our modified *in vitro* colony inhibition (CI) technique and the procedure for collecting peritoneal cells (PC) from mice were previously described [12–18]. Briefly, aliquots of 100 monodispersed target tumor cells, obtained from trypsinized suspensions, were seeded in 1 ml of medium into each of 24 wells of Disposo trays (Linbro Chemical Co., New Haven, Conn., U.S.A.). The plates were incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. The next day, PC were collected by peritoneal washings, using specially designed multi-perforated needles, from mice of experimental groups as well as from normal control mice, and 5×10^5 PC in 1 ml of culture medium were overlaid on the previously plated target cells. At least 4 parallel wells were used for each PC preparation. Twenty-four hours later, the culture media in wells were renewed and plates were reincubated for 5 more days. They were then stained with a 0·1% crystal violet solution and the number of colonies of tumor cells was counted under a stereomicroscope. The results were expressed as % CI by calculating the average number of colonies in the presence of PC from mice of experimental groups as compared with those in parallel wells exposed to an equal number of PC from normal control mice.

For *in vitro* blocking assays with sera from experimental and control mice, microcytotoxicity tests, using Falcon microplates (Falcon 3040), were performed as follows: one hundred tumor cells in 0·1 ml of culture medium were seeded in each microwell of the plates with the aid of Eppendord pipette. The plates were incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. The next day, the medium in wells was pipetted off and 0·1 ml of 1:3 diluted sera to be tested as well as 10^5 sensitized non-adhering PC in 0·1 ml of culture medium were added simultaneously. The non-adhering PC were used preferentially in order to avoid confusion with target tumor cells. The specifically sensitized PC were obtained by peritoneal washings from groups of 15–20 adult female C3HeB/Fe mice actively hyperimmunized with heavily irradiated (10,000 γ) TM1 tumor cells. To obtain from total PC the

fraction of non-adhering cells, freshly harvested whole PC were incubated at 37°C for 2 hr in Falcon Petri-dishes and the supernatants containing these cells collected after gentle agitation. The plates were incubated for 2 days then fixed in methyl alcohol and stained with 10% Giemsa solution. The number of remaining tumor cells on the bottoms of wells were counted under a stereomicroscope. Blocking activity was calculated by comparing the % cytotoxicity obtained after incubation with a test serum with parallel % cytotoxicity with control serum from normal mice. On the whole, quite satisfactory concordance was obtained when the CI and the microcytotoxicity tests were run parallely [17].

Statistical analyses

They were performed using the Student *t* test and differences were considered to be significant if $P \leqslant 0{\cdot}05$.

RESULTS

Effects of BCG on the in vivo *growth of TMI tumors*

Adult female C3HeB/Fe mice were divided into 3 groups. The mice of the first group (A) were injected intradermally (i.d.) on the left flank with a standard dose of 200 μG of BCG in 0·01 ml of saline. The remaining groups (B & C) were injected by the same route with 0·1 ml of saline. Seven days later, all mice were inoculated subcutaneously (s.c.) on the opposite flank with 10^6 TM1 cells, a dose which gave 100% tumor takes in normal syngeneic adult mice. At the 7th day after inoculation of tumor cells, mice of the group B received the standard dose of 200 μg of BCG whereas the mice of the other 2 groups received 0·1 ml of saline. All inoculated mice were checked twice a week for tumor development by measuring two perpendicular diameters with calipers.

The tumor growth in these 3 groups of mice is represented by the curves of Fig. 1. All 17 mice inoculated with TM1 tumor cells without treatment with BCG (group C) developed relatively rapidly growing tumors whose average diameter at day 35 attained 12 mm. The mice treated with BCG 7 days *before* inoculation of tumor cells (group A) showed obviously a slower tumor growth and the average diameter of their tumors at the corresponding day was only of 7 mm. Statistical analysis revealed that the difference was highly significant ($P < 0{\cdot}001$). Furthermore, complete regressions of the tumors were observed in 2 out of 10 mice of the latter group. On the contrary, the mice treated with BCG 7 days *after* inoculation of tumor cells (group B), at the time when small but palpable nodules were already present, have shown a rather accelerated growth if compared with the control untreated mice, though this difference was at the limit of significance.

Parallely, the same 3 groups of experimental and control mice were submitted to peritoneal washings regularly once a week starting from day 7 after inoculation of the tumor cells. Pooled PC from each group of mice were tested for their immunological activity by *in vitro* CI technique as described in Material and Methods.

As shown in Fig. 2, PC taken from the control group C mice, not treated with BCG, showed, as expected [12–15], a characteristic pattern of evolution of *in vitro* tested immunological activity. The PC inhibited significantly (40% CI at day 7) *in vitro* growth of the tumor target cells when taken from animals at the early stage of the tumor growth but have lost this activity almost completely at day 14, this state lasting until the death of the tumor bearing mice. The *in vitro* activity of PC from the group B mice, treated with BCG 7 days *after* inoculation of the tumor cells, showed a similar evolution toward a loss of cytotoxic activity showing even a significant inversion of this activity demonstrated as enhancement of growth of the tumor target cells (−33% CI at day 28). In contrast, PC from mice of the group A, treated with BCG 7 days *before* inoculation of the tumor cells, exhibited a very high cytotoxicity (77% CI) at day 14 and maintained this level up to day 35. Thus, a good correlation could be established between the effect of BCG on tumor growth *in vivo* and its effect on the evolution of specific cell-mediated antitumor immunity as revealed by *in vitro* tests.

In order to check for the possible effect of BCG on the evolution of serum blocking factor(s) supposedly capable to abrogate specific *in vitro* cytotoxicity, the mice of the above 3 groups were bled repeatedly every 2 weeks after tumor inoculation by tail cuttings and pooled sera of each group of mice were prepared and kept frozen for appropriate tests. Control normal sera were obtained from normal adult C3HeB/Fe mice. All sera were inactivated at 56°C for 30 min, diluted 1:3 with MEM and used for blocking assays. The results are represented on the graphs of Fig. 3. The sera obtained at day 7 from the group C mice having received TM1 cells only, abrogated significantly (16% blocking) ($P < 0{\cdot}05$) the cytotoxic activities of the sensitized PC. Later

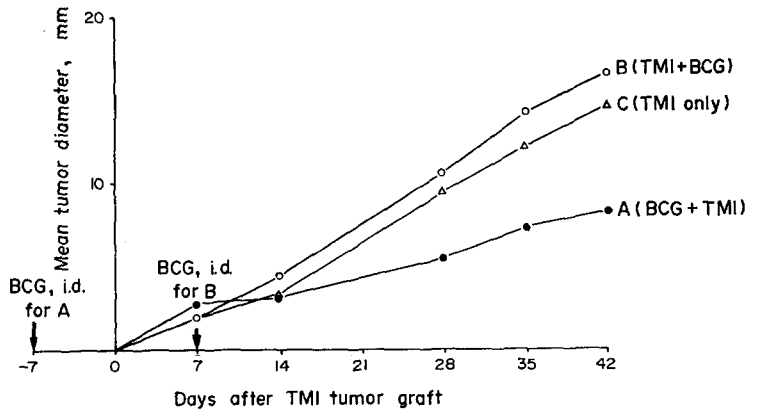

Fig. 1. In vivo *growths of TM*1 *tumors:*
Group A (●——●)*;* 10 *mice treated with BCG, i.d.* 7 *days before TM*1 *tumor graft.*
Group B (○——○)*;* 10 *mice treated with BCG, i.d.* 7 *days after TM*1 *tumor graft.*
Group C (△——△)*;* 17 *control TM*1 *tumor-bearing mice, not treated with BCG.*

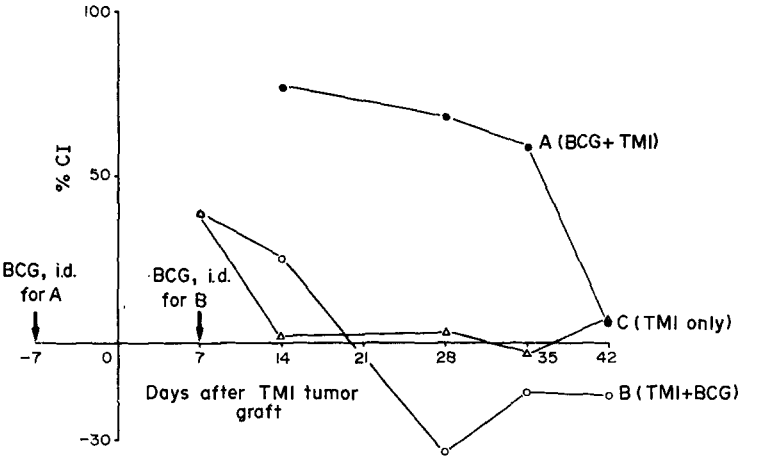

Fig. 2. In vitro *activity of PC from mice of:*
Group A (●——●)*;* 10 *mice treated with BCG, i.d.* 7 *days before TM*1 *tumor graft.*
Group B (○——○)*;* 10 *mice treated with BCG, i.d.* 7 *days after TM*1 *tumor graft.*
Group C (△——△)*;* 17 *control TM*1 *tumor-bearing mice, not treated with BCG.*

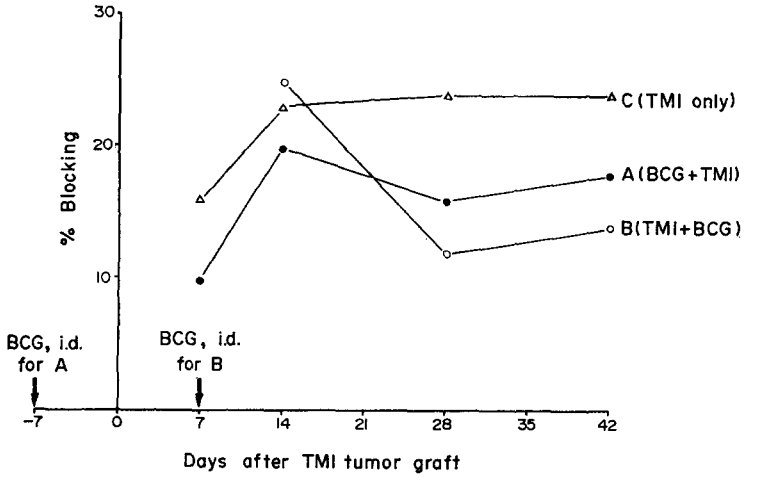

Fig. 3. In vitro *blocking activity of serum from mice of:*
Group A (●——●)*;* 10 *mice treated with BCG, i.d.* 7 *days before TM*1

Group B (○——○)*;* 10 *mice treated with BCG, i.d.* 7 *days after TM*1 *tumor graft.*
Group C (△——△)*;* 17 *control TM*1 *tumor-bearing mice, not treated with BCG.*

the levels of blocking factors progressively increased along with the tumor growth. However, the blocking activities of sera from mice of both groups A and B (treated with BCG) regardless of time relationships in respect to the tumor inoculation, were significantly lower than those of sera from the group C mice bearing tumors but non treated with BCG. Some variance of levels of serum blocking factor between the groups B and A was observed at days 28 and 42 but the differences were statistically hardly significant ($P < 0{\cdot}1$).

Effect of intratumoral (i.t.) BCG inoculation on the growth of TM1 tumors

The experimental design was as follows. Adult female C3HeB/Fe mice were divided into 4 groups, each comprising 12 mice. BCG, 200 μg in 0·2 ml of saline, was administered i.d. on the left flank of mice of 2 groups (groups a and b); 0·2 ml of saline was given to mice of the remaining 2 groups (groups c and d). Seven days later (day 0), all mice were inoculated s.c. on the opposite flank with 10^6 TM1 tumor cells. At day 28, the same standard doses of BCG were injected directly into tumors of mice of the groups a and c. Mice of the other groups received 0·2 ml of i.t. injection of saline. Tumor growth as well as *in vitro* CI activities of PC from these mice were checked regularly as in the previous experiments.

The evolution of tumor growth in these 4 groups of mice was represented by the curves in Fig. 4. The mice of the group b, which received BCG once i.d. 7 days before inoculation of the tumor cells, showed, in confirmation of the previous results, significantly ($P = 0{\cdot}05$) slower growth of their tumors than those of the control group d mice that did not receive BCG. Subsequent additional inoculation of BCG into the tumors at day 28, when the tumor size attained in average 7 mm in diameter (group a), resulted in further retardation of tumor growth. The average diameter of tumors at the 21st day after i.t. BCG inoculation (or day 49 after inoculation of the tumors) was 13 mm while that of tumors on the control group d mice was 18 mm ($P < 0{\cdot}01$). However, no regression of tumors was observed in mice treated by i.t. injection of BCG. The mice of the group c which received once i.t. BCG inoculation at day 28 showed also a slightly retarded growth of their tumor in comparison with that of the control group d mice. On the whole, the most pronounced and statistically highly significant retardation effect of tumor growth was obtained in presensitized mice receiving additional i.t. BCG inoculation.

The evolution of *in vitro* cell-mediated immunity in the above 4 groups of mice are shown in Fig. 5. PC from the group b mice that received BCG once 7 days before inoculation of TM1 tumor cells, exhibited, as in the previous experiments, earlier and longer lasting capacity to inhibit *in vitro* growth of the tumor target cells than those from the control group d mice not treated with BCG. If the BCG presensitized mice were reinoculated with BCG into their tumors at day 28 (group a), their PC showed significantly increased and accelerated inhibition (2% CI) at the 21st day after the second BCG challenge (or at day 49 after tumor inoculation). PC from the parallel mice which received only one sensitizing preinoculation of BCG (group b) showed very low activity (4% CI) at that time. However, PC from the group c mice, that received only i.t. BCG inoculation, without prior sensitization, revealed also an abrupt increased activity 21 days after BCG inoculation ($-18{\cdot}6$% CI at day 35 and 46% CI at day 49).

Comparative effects of BCG inoculated before or after surgical removal of tumor on the evolution of in vitro *cell-mediated immunity*

In order to investigate the most appropriate timing of BCG inoculation in relation to the surgical removal of the tumors, we have undertaken the following experiments. Adult female C3HeB/Fe mice were divided into 3 groups, each comprising 12 to 15 mice. All mice were inoculated s.c. with 10^6 TM1 tumor cells at day 0. At day 22, when the tumors measured 7–9 mm dia, the mice of the group I received BCG, 500 μg i.p. and 500 μg i.d. at the opposite site of tumor inoculation. The mice of the remaining groups II and III received similarly the same volume of saline. Seven days later (or at day 29 after tumor cell inoculation), all tumors were radically removed by surgical resection and BCG was inoculated similarly, 3 days later, into the operated mice of the group II. The mice of group III served as control (operated mice not treated with BCG).

All the operated mice were submitted to weekly peritoneal washings thereafter and their pooled PC were assayed by *in vitro* CI techniques. As shown in Fig. 6, the tumor cell inhibiting activity of PC from control operated mice, appeared significantly (42% CI) at day 16 after tumor operation and the same level of activity was maintained by day 24. However, PC from group I mice, that received BCG before tumor operation; attained a similar level of activity already at day 8 after tumor operation. This activity increased progressively

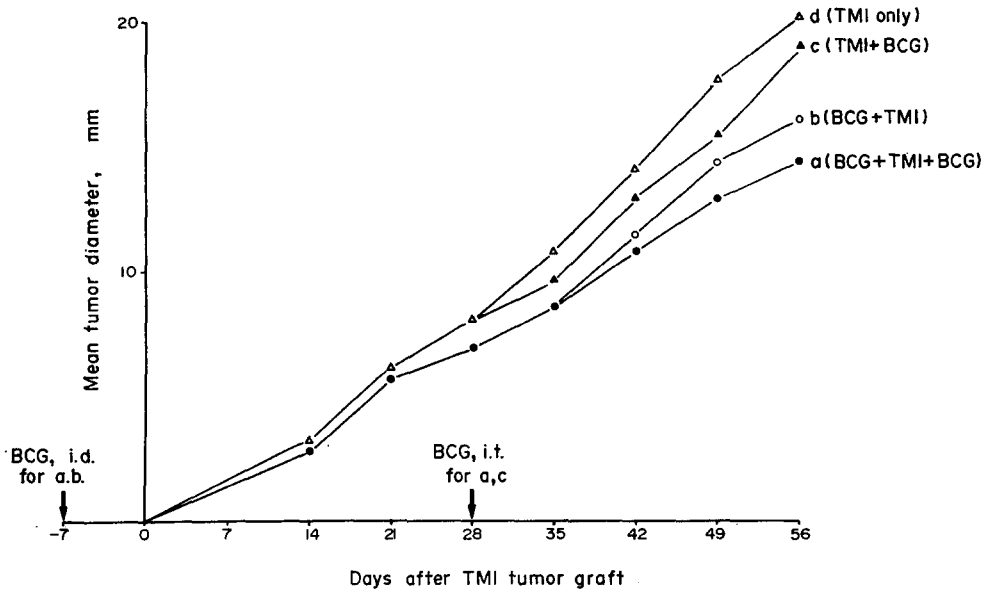

Fig. 4. In vivo *growths of TM*1 *tumors*:
Group a (●——●); 12 *mice treated with BCG, i.d.* 7 *days before and i.t.* 28 *days after TM*1 *tumor graft.*
Group b (○——○); 12 *mice treated with BCG, i.d.* 7 *days before TM*1 *tumor graft.*
Group c (▲——▲); 12 *mice treated with BCG, i.t.* 28 *days after TM*1 *tumor graft.*
Group d (△——△); 12 *control TM*1 *tumor-bearing mice, not treated with BCG.*

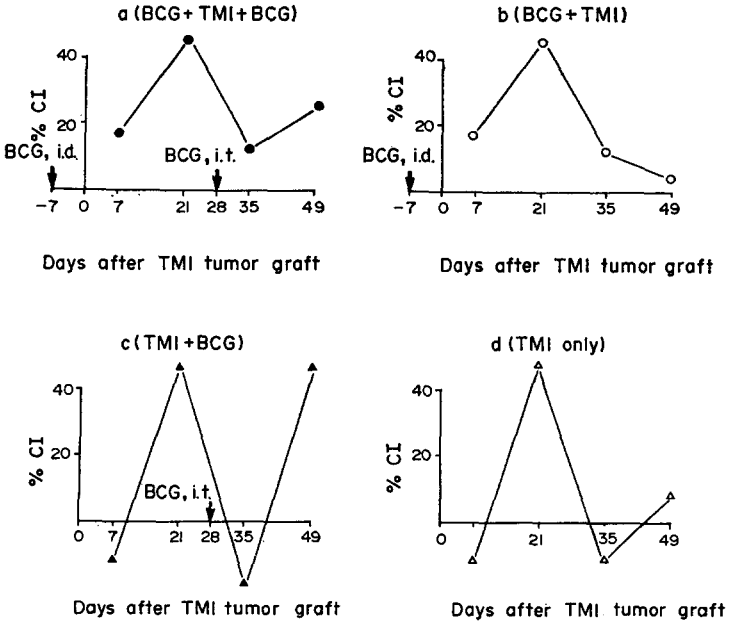

Fig. 5. In vitro *CI activity of PC from mice of:*
Group a (●——●); 12 *mice treated with BCG, i.d.* 7 *days before and i.t.* 28 *days after TM*1 *tumor graft.*
Group b (○——○); 12 *mice treated with BCG, i.d.* 7 *days before TM*1 *tumor graft.*
Group C (▲——▲); 12 *mice treated with BCG, i.t.* 28 *days after TM*1 *tumor graft.*
Group d (△——△); 12 *control TM*1 *tumor-bearing mice, not treated with BCG.*

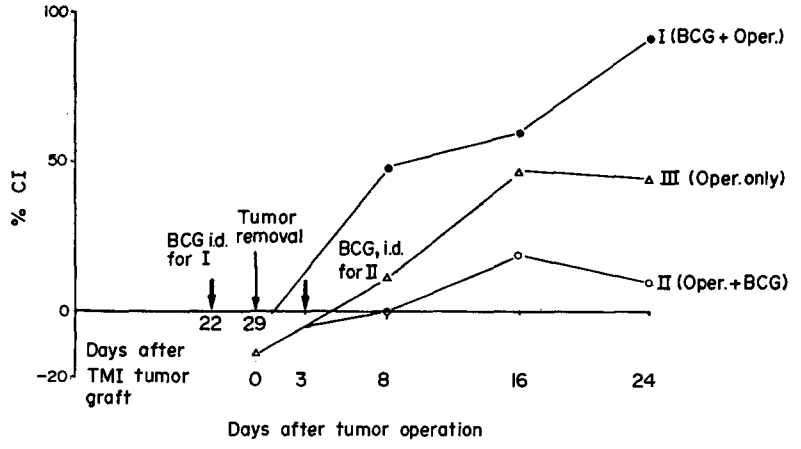

Fig. 6. In vitro *CI activity of PC mice of:*
Group I (●——●); 15 *mice treated with BCG, i.d.* 7 *days before TM*1 *tumor removal.*
Group II (○——○); 15 *mice treated with BCG, i.d.* 3 *days after TM*1 *tumor removal.*
Group III (△——△); 12 *control operated mice, not treated with BCG.*

reaching 83% CI at day 24. In contrast, immune reactivity of PC from group II mice that received BCG 3 days after tumor operation were found to be at particularly low levels. Thus, it is concluded that in this TM1 tumor system, BCG administration *before* tumor operation shortened significantly the post-operative eclipse period, whereas similar inoculation of BCG *after* tumor operation showe d a rather adverse effect on the post-operative recovery of the specific antitumor immunity of PC.

DISCUSSION

Since the first trials of Old *et al.* [1] and Halpern *et al.* [2], the treatment with living BCG was frequently applied with the aim to stimulate the immunological antitumor reactivity in experimental [3–5] and more recently in clinical malignancies [6, 7]. Different protocols of treatment were followed and the results visibly depended on various parameters corresponding to conditions of tumor growth and treatment by conventional methods, surgical removal of the tumor mass at the first place, as related to the timing and way of treatment with BCG.

In the here reported series of investigations we tried to answer some precise questions concerning the relationship between these parameters and the action of BCG. We used uniformly a C3HeB/Fe mammary tumor *in vitro* adapted TM1 cell line which was previously the subject of a series of experimental studies dealing with the evolution of cell-mediated immunity in syngeneic mice [14, 16, 18]. A quite stable tumor producing capacity and a rather weak but constant antigenicity as well as a quite uniform behaviour *in vitro* in terms of plating efficiency were characteristic for this cell line. This point was of importance since we tried throughout this whole study to establish in different circumstances a parallel between the rate of tumor growth or inhibition *in vivo* and the specific cell-mediated immunity as checked in the same animals by *in vitro* tests using lymphoid cells. These cells collected periodically by peritoneal washings were confronted with the TM1 target tumor cells.

From the first series of experiments we could conclude that the time of treatment with BCG with regard to the inoculation of TM1 tumor cells was essential. Injection of BCG by i.d. route 7 days *before* inoculation of tumor cells was efficient in terms of inhibition of tumor growth, whereas a similar inoculation of BCG 7 days *after* tumor graft, when the mice had already a palpable nodule, showed no effect or even enhanced their growth. These results were well correlated with those obtained from *in vitro* CI tests with PC of the same animals: from the beginning of the tumor growth inhibiting activity of PC from mice which received BCG *before* tumor graft was significantly higher than those of the other groups of mice and this elevated activity was still high at the 35th day when the PC from control mice or from mice that received BCG *after* tumor graft were entirely inactive.

Thus, our results confirm once more the "preventive" value of antitumor treatment with BCG operating most certainly by a non-specific stimulation of immunological reactivity.

These results were, as a whole, in concor-

dance with those obtained in another host–tumor system by Bansal and Sjögren [19]. These authors reported that BCG administration at the time of rat polyma tumor isografting, or 2 weeks before, inhibited tumor growth and induced an increased level of cell-mediated immunity whereas similar inoculation of BCG at the time when the tumor isograft had already grown out to a palpable nodule did not inhibit tumor growth and caused rather its enhancement.

Blocking activities of sera from mice, whether they received BCG *before* or *after* tumor graft, were lower in both groups than those of sera from the control tumor bearing mice and one can suppose that this effect may play a certain if not essential role in the activation of specific, cell-mediated immunity of the tumor bearing host. However, the flagrant difference in *in vivo* tumor growth depending on whether the BCG was given *before* or *after* inoculation of tumor cells did not appear in tests checking the serum blocking factors and it may be assumed that mechanisms other than those conditioning the presence of this factor (or factors) are involved in stimulation of cell-mediated antitumor immunity by BCG.

In our expeiments in which BCG was inoculated directly into the growing tumors, a significant retardation of the tumor growth has been observed not unlike that reported by other authors [3, 5, 20, 21]. The *in vitro* PC-mediated specific antitumor immunity of these mice showed concurrently an abrupt increase around 2 weeks after the BCG inoculation, while that of the control non treated mice was still at a rather low level of activity. Thus again a satisfactory correlation was observed between results obtained *in vivo* and *in vitro*. It is remarkable that in mice preimmunized with BCG the tumor inhibition effect following i.t. inoculation of BCG was much more pronounced. Increased inhibition of tumor growth by such preimmunization before i.t. inoculation of BCG was reported also in a mouse fibrosarcoma [22]. Nevertheless, no complete regression of tumor was observed even in presensitized animals in our experiments. Tumor size at the moment of intratumoral inoculation of BCG seemed to be a critical limitation for successful effect of immunostimulating adjuvants [21]. In our experiments BCG was inoculated into tumors measuring approximately 8–10 mm dia. It was reported elsewhere [23] that an autochthnous methylcholanthrene-induced murine fibrosarcoma of less than 8 mm dia was more susceptible to regression induced by BCG than were tumors of larger size [23].

The third series of experiments was undertaken in order to find out optimal time-relationship between administration of BCG and surgical removal of tumors as expressed particularly in terms of the evolution of cell-mediated immunity checked by *in vitro* tests. Clearly, in the used system, the administration of BCG, 7 days *before* tumor operation, resulted in shortening of the immunological eclipse period in this tumor–host system.

Concordantly, Smith *et al.* [24] demonstrated that in another host–tumor system (a guinea pig hepatoma) i.d. injection of BCG *before* local excision of the tumor eradicated lymph node metastases. On the other hand, Sparks *et al.* [25] have shown that administration of BCG both *before and after* surgery were effective in inhibiting growth of a syngeneic mammary adenocarcinoma in rats. So, it seems premature to generalize the indication appearing clearly in our C3H mouse–TM1 tumor system that pre-operative treatment with BCG is generally recommendable and efficient in stimulating the anti-tumor immune reaction. As a matter of fact, in a limited number of experiments (unpublished data) performed with BALB/c mice and a syngeneic tumor cell line, chronically infected with a low leucemogenic murine leukemia virus variant, we observed that the immunological eclipse state was not shortened but, on the contrary, prolonged in mice treated with BCG one week before surgery as compared with operated, non treated animals. These results which seem to branch off from the others may be due to the particular character of the chronically virus-infected tumor cell line.

Further work using different host-tumor models and correlating *in vivo* and *in vitro* results is necessary to supply clear and unequivocal indications concerning the most beneficial way and timing of treatment with BCG or other immunostimulants. The here reported results indicate that the judicious choice of the protocol of this treatment is of paramount and critical importance.

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Plasma Androstenedione Levels in Women with Breast Cancer

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Abstract—*The levels of plasma androstenedione have been measured in 79 normal women, 65 and 61 patients with early breast cancer (Stage I or II) one day before and ten days after mastectomy, respectively, and 69 patients with advanced metastatic disease.*

There was no significant difference between the mean levels of androstenedione in normal women and subjects with early breast cancer, before mastectomy. However, the general level of androstenedione was significantly subnormal after mastectomy, or in patients with advanced breast cancer. Also there was a significant reduction in the general level of plasma androstenedione associated with mastectomy.

In all categories of women the plasma level of androstenedione was highly correlated with the plasma concentrations of dehydroepiandrosterone sulphate and the excretion of urinary 11-deoxy-17-ketosteroids.

INTRODUCTION

THERE is now considerable literature dealing with androgen status in women with an enhanced risk of breast cancer or in patients with the disease [1–9]. The general finding is that urinary and plasma androgens are subnormal [10–16] but normal [17–19] or high [20] values have also been reported.

We have recently found that plasma androgen sulphates [16] and dehydroepiandrosterone (DHA) [21] are subnormal in women with either early or advanced breast cancer and have now measured the plasma levels of androstenedione.

MATERIAL AND METHODS

Subjects

The 79 ostensibly healthy women volunteers (mean age 48·7 yr) constituted the normal control group. The patients with early breast cancer (Stage I or II) were 65 (mean age 51·5) and 61 (mean age 50·9) women from whom blood was taken one day before and 10 days after mastectomy, respectively. There were 69 patients (mean age 50·9) with advanced disseminated disease. Plasmas from heparinised bloods from these women were stored at −20°C until analyzed.

Androstenedione measurement

Plasma (0·2 ml) was shaken with 2 ml hexane for 15 minutes and centrifuged (700 *g*) for 10 min. Aliquots (0·2 ml) of the hexane layer were evaporated and borate buffer (pH 8·0; 0·05 M), antibody (final concentration 1:10,000) and [1·2-H^3] androstenedione (4000 dis/min; 10 pg) added and the mixture (final volume 0·2 ml) incubated overnight at 4°C. Dextran coated charcoal was added, shaken and the assay tubes left in an ice-bath for 15 min, after which the tubes were centrifuged (700 *g* × 10 min) and aliquots of the supernatant counted in a liquid-scintillation counter. The amounts of androstenedione were estimated from a standard curve and the results corrected for 78% recovery. This recovery figure was arrived at by estimating the recovery of tritiated androstenedione from plasma at various times and with different batches of hexane. The coefficients of variation for 12 such estimations was 2·5%. Hexane blanks and quality control plasmas were included in each run to test for non-specific interference and reproducibility of the assay, respectively.

The antibody to androstenedione was raised

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in rabbit using androstenedione conjugated in the 6 position to bovine serum albumin. The cross reaction of this antibody to steroids likely to be present in plasma in appreciable concentrations was negligible except for testosterone and 11β-hydroxyandrostenedione, which cross-reacted at a level of less than 5%. Under the conditions of the assay only about 20% of testosterone or 11β-hydroxyandrostenedione are extracted by hexane. Thus the addition of a large amount of 11β-hydroxyandrostenedione to plasma, equivalent to 800 ng/100 ml, resulted in an apparent increase in androstenedione of 17 ng/100 ml (125 ng to 142 ng/100 ml).

Assay precisions were measured by comparing duplicate estimations and gave coefficients of variation of 8%, 6% and 7% for ranges of androstenedione of 0–50, 51–100 and greater than 101 ng per 100 ml plasma, respectively. There were 20 duplicate estimations in each group.

Plasma dehydroepiandrosterone sulphate (DS) and androsterone sulphate (AS) were measured by gas–liquid chromatography by the method of Wang *et al.* [9, 22]. The urinary 11-deoxy-17-ketosteroids (11-DKS) were determined in normal women and patients, 10–14 days after surgery, with early breast cancer using the method outlined by Bulbrook *et al.* [12].

RESULTS

The mean levels of plasma androstenedione in normal women, patients with breast cancer before and after mastectomy and those with advanced breast cancer are shown in Fig. 1. The elevation of the regression line of steroid on age for normal women is significantly higher than that for patients with advanced breast cancer ($F = 8{\cdot}36$; $P < 0{\cdot}005$) and for patients with early breast cancer after mastectomy ($F = 33{\cdot}4$; $P < 0{\cdot}001$). But it is clear from Fig. 1 that the mean level before mastectomy is not significantly different from that found in normal women. A similar result is obtained when the data are analysed with reference to menopausal status. The mean levels in pre- or post-menopausal controls and in the three groups of patients with breast

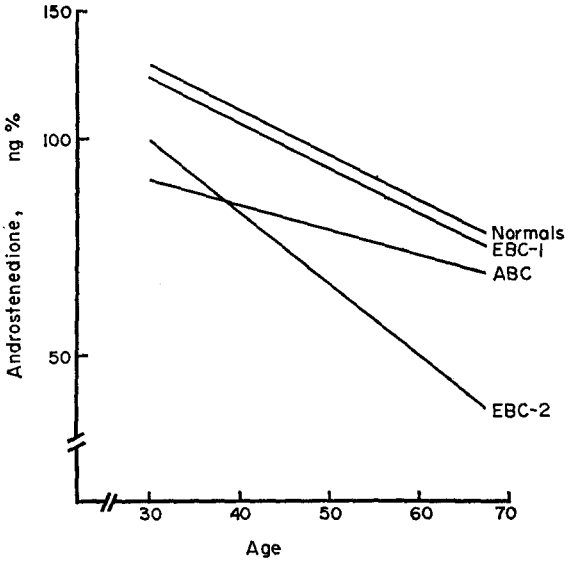

Fig. 1. *Plasma androstenedione levels in normal women and patients with breast cancer. EBC-1 and EBC-2 refer to women with early breast cancer from whom blood was taken one day before and* 10 *days after mastectomy, respectively. ABC refers to women with advanced breast cancer.*

Table 1. *Mean plasma androstenedione levels in pre- and post-menopausal women*

	Pre-menopausal	Post-menopausal
Control	2·016 ± 0·124 (39)	1·929 ± 0·108 (40)
Early breast cancer Before mastectomy	1·980 ± 0·260 (36)	1·915 ± 0·252 (29)
t;P	0·78 N.S.	0·32 N.S.
Early breast cancer After mastectomy	1·855 ± 0·291 (34)	1·672 ± 0·242 (27)
t;P	3·17 $P < 0{\cdot}001$	5·91 $P < 0{\cdot}001$
Advanced breast cancer	1·887 ± 0·358 (33)	1·827 ± 0·290 (36)
t;P	2·12 $P < 0{\cdot}05$	2·07 $P < 0{\cdot}05$

Results are expressed as log mean (ng/100 ml) ± S.D. with the number of subjects in parenthesis. "t" and "P" refer to the comparison with the mean normal levels.

cancer are given in Table 1. The pre-mastectomy levels of androstenedione do not differ from those found in the controls but the concentration of this steroid is significantly decreased after mastectomy and in the advanced disease.

Mastectomy itself is associated with a decrease in androstenedione and in 44 patients from whom blood was obtained before and after mastectomy a comparison by paired *t*-test showed this decrease to be highly significant ($t = 4{\cdot}76$; $P < 0{\cdot}001$).

Relationship between plasma androstenedione and plasma DS, AS and urinary 11-*DKS*

(*a*) *Plasma DS and AS.* In all the groups studied the amounts of plasma androstenedione are highly correlated ($P < 0{\cdot}001$) with plasma DS levels (both concentrations expressed logarithmically). In controls and patients with early breast cancer before surgery, the levels of both plasma DS and androstenedione are significantly correlated with age (Reference [16] and Table 2). Calculation of the partial correlation coefficient [23] for these categories (Table 2) shows that the significant correlation between DS and androstenedione is independent of age. In contrast to the high degree of correlation between androstenedione and DS, in all categories of women, the correlation between androstenedione and AS reached formal significance levels in only normal control women ($P < 0{\cdot}05$) and patients with advanced breast cancer ($P < 0{\cdot}01$) (Table 2).

(*b*) *Urinary* 11-*DKS.* The concentrations of plasma androstenedione are significantly correlated with the amount of urinary 11-DKS excreted for normal women and in both groups of patients with early breast cancer (Table 2). Since the amount of urinary 11-DKS excreted is correlated with age [16] the partial correlation coefficient has been calculated, where plasma androstenedione is also correlated with age, and shows that the significant relationship between plasma androstenedione and urinary 11-DKS is independent of age.

DISCUSSION

There is now sufficient evidence in the literature to assemble a persuasive argument that androgen metabolism is abnormal in women with breast cancer. Thus urinary androgen metabolites are subnormal in women with early and advanced breast cancer and this is reflected by low levels of plasma DS, AS and DHA [10–16, 21]. This present report confirms and extends the results of Poortman *et al.* [24–26] and shows that the general level of plasma androstenedione is significantly subnormal in women with early breast cancer ten days after surgery, and patients with advanced breast cancer. Furthermore, these subnormal levels of plasma androstenedione are not a

Table 2. *Correlation between androstenedione and age, DS, AS and* 11-*DKS*

	x	*y*	Intercept	Slope	*N*	*r*	*P*	*r**	*P**
Controls	Age	log Δ^4	2·28	−0.0060	79	−0·50	<0·001		
	log DS	log Δ^4	1·54	0·236	79	0·51	<0·001	0·42	<0·001
	log AS	log Δ^4	1·86	0·084	79	0·22	<0·05		
	log 11-DKS	log Δ^4	1·36	0·180	77	0·36	<0·01	0·27	0·02
Early breast cancer (pre-op)	Age	log Δ^4	2·27	−0·0062	65	−0·20	NS		
	log DS	log Δ^4	1·17	0·446	65	0·49	<0·001		
	log AS	log Δ^4	1·66	0·216	65	0·23	NS		
	log 11-DKS	log Δ^4	1·18	0·229	59	0·32	<0·02		
Early breast cancer (post-op)	Age	log Δ^4	2·30	−0·0100	61	−0·32	<0·02		
	log DS	log Δ^4	0·94	0·536	61	0·52	<0·001	0·46	<0·001
	log AS	log Δ^4	1·68	0·105	61	0·11	NS		
	log 11-DKS	log Δ^4	1·14	0·196	61	0·62	<0·001	0·56	<0·001
Advanced breast cancer	Age	log Δ^4	2·05	−0·0035	69	−0·10	NS		
	log DS	log Δ^4	0·94	0·567	69	0·62	<0·001		
	log AS	log Δ^4	1·60	0.238	69	0·36	<0·01		

*r** = partial correlations coefficient (Snedecor); *P** indicates that the relationship between the parameter is independent of age. Androstenedione is expressed as ng/100 ml and DS and AS as μg/100 ml. The excretion of 11-DKS is expressed as mg/24 hr.

result of abnormalities in plasma protein binding in women with breast cancer [27].

Androstenedione levels before mastectomy in women with early breast cancer were not significantly abnormal, and this difference between pre- and post-operative patients stems from the changes in plasma androstenedione associated with mastectomy.

A similar alteration has been reported for plasma DS and AS levels and the urinary excretion of 11-DKS [16]. It is not possible with the present data to assess how near either the pre- or post-operative androstenedione levels are to basal levels. It is possible that the trauma of surgery could result in a decreased level, whilst it is equally possible that stress results in an increase in pre-operative levels (see 16). Since plasma androstenedione levels in patients with early breast cancer are significantly correlated with the amount of urinary 11-DKS excreted, and the level of urinary 11-DKS is subnormal in women who subsequently develop clinical breast cancer [3, 4], it would appear that the post-operative levels approximate to a non-stressed basal level.

It will be noticed that in the statistical analysis of androstenedione levels in pre-menopausal women, no account is made for the stage of the cycle. The justification for this stems from the large between-person variation found in androstenedione levels. Our experience in not being able to detect differences in mean androstenedione levels in the luteal phase and other parts of the cycle are similar to that of Horton [28]. In fact, the changes in androstenedione levels during the menstrual cycle, as reported by Baird *et al.* [29], was only demonstrable if serial blood samples from the same subjects are analyzed.

This study shows that in addition to the urinary 11-DKS, plasma DS, AS and DHA [10–16, 21], plasma androstenedione levels are also generally low in women with breast cancer. However, the greatest difference occurred in post-menopausal women. Since the ovaries can contribute 20–50% of the blood production rate of androstenedione, depending on the stage of the menstrual cycle [30, 31], it is possible that any abnormality in the adrenal production of androstenedione in pre-menopausal women could be obscured by such a substantial ovarian production. Certainly the results on plasma DS [16] would suggest that the abnormality in androgen status in breast cancer patients is of adrenal origin, and this conclusion is supported by the significant correlation between the levels of plasma DS and androstenedione. There is, in fact, no conclusive evidence that ovarian hormone production is abnormal in patients with breast cancer, and measurements of plasma oestrogen and progesterone levels have generally shown that these hormones are within the normal range in women with breast-cancer or with a high risk of breast cancer [7, 32–35]. Also, no abnormalities have been found in the conversion of androstenedione to oestrone in women with breast cancer [24–26]. If further work substantiate these findings, then it would appear that the main endocrine abnormality in breast cancer is of adrenal rather than of ovarian origin.

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Letter to the Editor

Comments on Yerushalmi's Article (Reference 1)

E. R. ATKINSON

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YERUSHALMI has recently reported that the treatment of tumors by whole body hyperthermia (WBH) led to more rapid metastasis than in untreated controls. He also reports that local hyperthermia led to a delay in metastasis. These conclusions are based on experiments with transplanted lewis lung carcinoma. Relevance to clinical WBH for the treatment of patients with metastatic tumors was indicated [1].

Yerushalmi appears, as have previous investigators [2], to have difficulty producing tumor temperatures above 41·5°C with WBH. In what he describes as WBH, tumor temperatures barely exceed 41·5°C for a few minutes, at most, but do involve maintenance at less than 41·0°C for periods of about twenty minutes. In what he describes as local hyperthermia, tumor temperatures exceeded 43·5°C and were maintained at temperatures in excess of 42·0°C for over twenty minutes. Thus, whole body temperatures of therapeutic interest were not employed although they appear to be well within the critical thermal maxima (ca. 43·5°C) reported for mice [3].

In view of data indicating a sharp threshold for tumor cell death beginning at about 41·5°C (see, for example, reference 4), it appears that Yerushalmi's results were simply the product of inadequate treatment by WBH paralleled with perhaps excessive treatment by local hyperthermia. The precision of ±0·5°C which Yerushalmi reports for his temperature measurements [5], however, casts serious doubt on any experimental conclusions.

Accepted 9 September 1976.

We have been investigating the supposition that inadequate hyperthermia treatment, consisting of prolonged exposure to temperatures less than 41·5°C, leads to increased tumor proliferation *in vivo*. Our work employs both local and WBH and involves the lewis lung carcinoma as well as other transplantable tumors with differing mechanisms of metastasis. To the extent that we can interpret Yerushalmi's data they appear to be in substantial agreement with our own but we differ greatly on what may be concluded from these data.

In our experiments, we employ careful confinement of endogenous metabolic heat by regulated air flow with control of evaporative losses, to produce WBH. In order to produce local hyperthermia we employ electromagnetic radiation of known, limited tissue penetration. In this manner, transcutaneous heat flow is kept small in both instances since it is electromagnetic radiation, not heat, which passes through the skin. It is difficult for us to see the relevance of air temperature, somewhere else in the apparatus, which Yerushalmi ascribes to *in vivo* hyperthermia and uses to compare local and WBH effects [1]. For this reason, we employ the output of tissue implanted temperature transducers to feed back tissue temperature controls with a precision of ±0·05°C.

We feel that the lowest temperature attained by tumor cells in a treatment regime, rather than the highest, could be an important indicator of treatment quality. It would be most unfortunate if this indicator were confused with anatomical extent of treatment, as Yerushalmi has done, prior to its careful validation.

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Announcements

FIRST SYMPOSIUM AND TASK FORCE ON: EXPERIMENTAL APPROACHES TO THE TREATMENT OF GASTROINTESTINAL TUMORS

This symposium, organized by the EORTC Gastrointestinal Tract Cancer Cooperative Group, will be held in Brussels on 14–15 April, 1977.

Three topics will be discussed:

1. Experimental models in G.I. tract oncology, excluding hepatoma.

2. Precancerous states of the G.I. tract.

3. Clinical trials in G.I. tract cancers.

Number of participants will be limited to 200. Abstracts (in English) of free communications must be submitted by 1 March, 1977. For details and registration, write to: Dr. M. Staquet, EORTC Data Center, Institut Jules Bordet, rue Héger-Bordet 1, 1000 Bruxelles, Belgium. The symposium will be held 2, rue Evers, 1000 Brussels. Registration fee: 2500 Belgian Francs (to be paid in advance).

PAG SYMPOSIUM ON SAFETY OF SINGLE CELL PROTEIN FOR ANIMAL AND HUMAN FEEDING

(31 *March and* 1 *April* 1977)

This technical symposium, sponsored by the Protein-Calorie Advisory Group of the United Nations System will be held at the Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62 - 20157 Milano Italy. Telephone: (02) 35.54.546 and 35.70.546; Telex: 37268 NEGRINST.

This International Symposium is being planned and will be held with the full cooperation and support of the "Mario Negri" Institute for Pharmacological Research. The proposed program consists of four technical sessions devoted to scientific discussions on Toxonomy and Pathogenicity, Metabolism and Safety of Alkanes and Odd Numbered Carbon Chain Fatty Acids (2 sessions), and Allergic Reactions in Man. Each invited speaker has played a key role in the area of investigation he will cover. The working language of the Symposium will be English.

There is no registration fee for attendance and participation in the symposium discussions. For more details please contact: Dr. Silvio Garattini, Director of Institute and Organizing Joint Secretary of the Symposium at the above address.

SECOND CONFERENCE ON LUNG CANCER TREATMENT

Airlie House, Virginia

(22–24 *May*, 1977)

This conference is sponsored by the Cancer Therapy Evaluation Program of the National Cancer Institute. Current trends in the treatment of lung cancer will be analyzed and prospects for further therapeutic approaches will be discussed. Workshops are planned for the following topics: pathology (chairmen: M. Matthews and R. Yesner), surgery (C. Mountain), adjuvant chemotherapy (G. Bonadonna), adjuvant immunotherapy (R. Ritts), staging and followup (H. Hansen), radiotherapy (C. Perez), chemotherapy of non-small cell carcinoma (P. Alberto and M. Cohen), small cell carcinoma (Y. Kenis and J. Minna), tumor immunology (P. Chretien), experimental chemotherapy and kinetics (F. Schabel), and new modalities (P. Rubin). Each workshop will be introduced with invited papers. Some papers will also be selected from submitted abstracts. Attendance will be limited to 120 persons. There is no registration fee.

For further information contact Dr. Franco M. Muggia, Second Conference on Lung Cancer Treatment, National Cancer Institute, Building 37, Room 6A17, Bethesda, Maryland 20014.

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Yu. S. Tatarinov and A. V. Sokolov: Development of a radioimmunoassay for pregnancy-specific $beta_1$-globulin and its measurement in serum of patients with trophoblastic and non-trophoblastic tumours.

N. W. Choi, P. T. Shettigara, H. A. H. Abu-Zeid and N. A. Nelson: Herpesvirus infection and cervical anaplasia—a seroepidemiological study.

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J. E. Byfield, M. Lynch, F. Kulhanian and P. Y. M. Chan: Cellular effects of combined Adriamycin and X-irradiation in human tumor cells.

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G. R. Shellam: Gross-virus-induced lymphoma in the rat. V. Natural cytotoxic cells are non-T cells.

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Effects of Oestradiol-17β and Tamoxifen on Nuclear Oestradiol-17β Receptors in DMBA-Induced Rat Mammary Tumours

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Abstract—*An exchange assay has been established for the measurement of nuclear oestradiol-17β binding sites in DMBA-induced mammary tumours of the rat. The assay was used to examine the nuclear concentration of binding sites following the administration* in vivo *of tamoxifen and oestradiol-17β. Both compounds translocate receptor protein to the nucleus and cause an early elevation in cytoplasmic receptor levels. By 24 and 48 hr after tamoxifen administration, the intranuclear concentration of oestrogen receptor complex has returned to levels indistinguishable from pretreatment values. At this time, replenishment of the cytoplasmic receptor had not occurred and tumours were non-responsive to oestradiol-17β.*

INTRODUCTION

THE INITIAL event in the antioestrogenic action of tamoxifen (ICI 46474; trans 1-(p-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene) on mammary tumours induced in rats by 7,12-dimethylbenz(a)anthracene (DMBA) is the reduction of both total [1] and accessible [1–3] cytoplasmic binding sites for oestradiol-17β *in vitro*. Although the final result of tamoxifen administration i.e. tumour regression [2, 4] is obvious, the precise sequence of events leading to this end-point remains unclear. This study examines the nuclear uptake of oestradiol-17β and tamoxifen as determined by an exchange assay, with the aim of providing further information as to the means by which antioestrogens can influence tumour growth.

MATERIAL AND METHODS

Animals

Mammary tumours were induced in virgin female Sprague–Dawley rats (50 ± 2 days old) by intubation with a single dose of DMBA (20 mg in 1 ml sesame oil). Animals were housed in groups of four and allowed diet and water *ad libitum*. Beginning 5 weeks after intubation with DMBA, rats were palpated for tumours at weekly intervals and tumour size recorded as the mean of two perpendicular diameters, one measured across the greatest width. Tumours which did not attain an approximate size of 20 mm mean diameter in the 23-week period following carcinogen administration were not used in the study.

Preparation of nuclear fractions and determination of oestradiol-17β/tamoxifen binding sites by the [^{3}H]-oestradiol-17β exchange assay

Animals were given oestradiol-17β (5 μg) or tamoxifen (100 μg) in 100 μl vehicle (10% v/v ethanol in 0·15 M NaCl) by intravenous injection 1 hr before sacrifice. The tumours were removed and dissected free of fat, washed with 0·25 M sucrose (adjusted to pH 7·4 with $NaHCO_3$), cut into small pieces and passed through a stainless steel press (pore diameter, 1·5 mm). The brei was collected in a beaker containing 0·25 M sucrose and homogenised with a Potter–Elvehjem glass-on-teflon homogeniser of clearance 14–16 μm (6 strokes, speed 2500 rev/min). Such a procedure has been demonstrated to give maximum breakdown of tumour cell membranes with minimal damage to cell organelles [5]. A nuclear preparation was

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obtained by centrifugation of the homogenate at 800 *g* for 10 min at 4°C. Nuclei were washed twice by resuspension and resedimentation from 0·25 M sucrose-3 mM $MgCl_2$ containing 0·1% (w/v) triton X-100, then washed and resuspended in 0·25 M sucrose-3 mM $MgCl_2$. Aliquots of the nuclear fraction (200 μl) were mixed with an equal volume of protamine sulphate [1 mg/ml in medium A (50 mM Tris-HCl buffer, pH 7·4, containing 5 mM EDTA and 1 mM dithiothreitol)] and re-sedimented at 800 *g* for 10 min. Precipitated receptor was incubated for various times at various temperatures (see Results) with 200 μl [2,4,6,7-^{3}H]oestradiol-17β (specific radioactivity 85 Ci/m-mole; Radiochemical Centre, Amersham, Bucks., U.K.) in medium A (final steroid concentration 20 nmole/l), in the absence or presence of unlabelled diethylstilboestrol (20 μmole/l). After incubation precipitates were cooled to 4°C and washed with cold medium A (three washes, each followed by centrifugation at 800 *g* for 10 min). The final pellet was extracted overnight with ethanol (0·5 ml), centrifuged and the ethanolic supernatant decanted into vials. The extracts were dried and counted in 6 ml scintillation fluid (4 g, 2,5-diphenyloxazole, 0·05 g of 1,4-di [2-(5-phenyloxazolyl)]-benzene, 1,1-toluene) in a Nuclear Chicago (Mark II) liquid scintillation spectrophotometer using external standardisation.

In other experiments, animals were injected with 20 μCi [^{3}H]oestradiol-17β 1 hr prior to removal of tumours. Under these conditions approximately 70% of the tumour-associated radioactivity was found in the nuclear fraction. Aliquots of the nuclear fraction (200 μl) were treated with protamine sulphate as previously described, centrifuged and the precipitated receptor incubated with a saturating concentration of either oestradiol-17β (20 nmole/l) or tamoxifen (20 nmole/l) at 4, 10, 15, 25 or 37°C for periods up to 4 hr.

Saturation analysis of the binding of [^{3}H] oestradiol-17β to otherwise occupied nuclear sites was estimated by incubating aliquots of the protamine sulphate precipitated receptor in medium A containing various concentrations of [^{3}H]oestradiol-17β (1–25 nmole/l) in the presence and absence of a 1000-fold higher concentration of unlabelled diethylstilboestrol for 2 hr at 15°C. After incubation tubes were treated as previously described.

The method for the determination of accessible cytoplasmic oestradiol-17β binding sites has been described in a previous communication [1]. Briefly, the supernatant from the 800 *g* centrifugation stage was subjected to further centrifugation at 105,000 *g* for 45 min at 4°C. Aliquots of cytosol were incubated for 16 hr at 4°C with equal volumes of medium A containing a saturating concentration of [^{3}H]-oestradiol-17β (5 nmole/l) in the presence and absence of a 100-fold higher concentration of unlabelled diethylstilboestrol. Excess steroid was removed by charcoal adsorption and aliquots of the charcoal-free supernatant counted for radioactivity.

For competitive binding studies various amounts of non-radioactive oestradiol-17β or other potential ligands (25–10,000 nmole/l) were added with [^{3}H]oestradiol-17β (either 5 nmole/l for accessible cytoplasmic binding sites or 20 nmole/l for nuclear binding sites) to the reaction mixture and incubated as described previously. All values were corrected by subtraction of the binding value obtained in the presence of a 1000-fold excess of unlabelled oestradiol-17β.

The DNA content of nuclear fractions and the protein content of cytosol fractions were estimated using the methods of Burton [6] and Lowry *et al.* [7] respectively.

Determination of nuclear and cytoplasmic binding sites by the [^{3}H]*oestradiol-17β-exchange assay following the* in vivo *administration of either oestradiol-17β or tamoxifen*

Three experiments were undertaken.

(i) Eighteen rats bearing 25 tumours were divided randomly into five groups (5 tumours per group). Each group received either oestradiol-17β (0·5 μg or 5μg) or tamoxifen (10 μg or 100 μg) in 0·1 ml vehicle [10% (v/v) ethanol in 0·15 M NaCl] by intravenous injection. Control animals received vehicle alone. Tumour biopsy samples (approximately 200 mg tissue) were removed aseptically from Nembutal ® anaesthetized animals at time 0 (prior to injection) and at 18, 25, 40 and 60 min after injection. Cytosol and nuclear preparations were obtained as previously described and portions incubated for either 2 hr at 15°C (total nuclear sites) or 16 hr at 4°C (accessible cytoplasmic sites) with a saturating concentration of [^{3}H]oestradiol-17β (20 nmole/l) with and without unlabelled stilboestrol (20 μmole/l).

(ii) Fifteen animals were divided randomly into three groups (5 tumours per group). The experimental design was as described in (i) except that animals were treated with either oestradiol-17β (5 μg), tamoxifen (100 μg) or vehicle alone and tumour tissue was biopsied at time 0, and at 0·5, 1, 2, 4, 8, 16, 24 and 48 hr after injection.

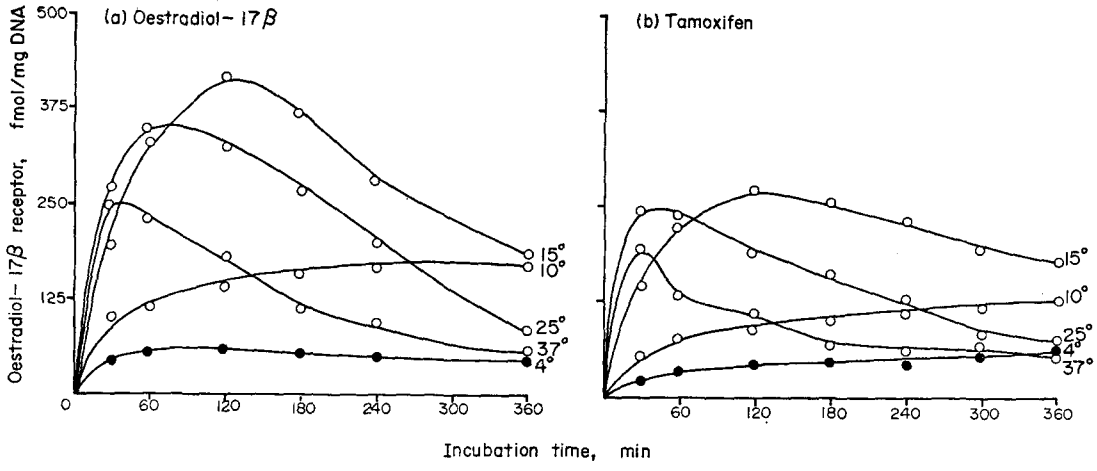

Fig. 1. Determination of binding sites—effect of temperature. Animals were given (a) *oestradiol-17β* (5 μg) *or* (b) *Tamoxifen* (100 μg) 1 *hr before sacrifice. Tumour nuclear preparations were incubated at* 4, 10, 15, 25 *and* 37°C *for periods up to* 360 *min with either* (1) *a saturating concentration of* [^{3}H] *oestradiol-17β* (20 *nmole/l*) *or plus* [^{3}H]*-oestradiol-17β* (2). *Plus a* 1000*-fold excess of unlabelled diethylstilboestrol. Specific oestradiol-17β binding was obtained by subtraction of* (2) *from* (1).

(iii) Animals were treated as follows: Group 1, 4 animals bearing six tumours received injections of tamoxifen (100 μg) on two consecutive days. After a further 24 hr, animals were given oestradiol-17β (5 μg). Group 2 animals (four animals bearing five tumours) received saline injections for the first two days followed by oestradiol-17β (5 μg) on the third. Group 3 animals (three animals bearing five tumours) received saline injections alone. Tumour biopsy samples were removed at time 0, 24, 48, 52, 56 and 60 hr and assayed for cytoplasmic and nuclear oestradiol-17β binding sites.

RESULTS

Measurement of nuclear oestradiol-17β binding sites

The establishment of conditions for the exchange of [^{3}H]oestradiol-17β with endogenous oestradiol-17β or tamoxifen specifically bound to nuclear oestradiol-17β receptors involved (a) determination of the optimum temperature to ensure the greatest rate of exchange with the minimum rate of degradation of binding sites and (b) reduction of the interference of non-specific binding components.

(a) *Temperature optimisation of incubation conditions.* Nuclear fractions prepared from mammary tumours pretreated *in vivo* with oestradiol-17β contain elements that bind [^{3}H]oestradiol-17β (Fig. 1a). Rapid uptake of [^{3}H]oestradiol-17β was observed at 15, 25 and 37°C. Continued incubation of the receptor complex resulted in thermal inactivation. This phenomenon was most evident at 25 and 37°C. Incubations of nuclear fractions at 4°C caused little [^{3}H]oestradiol-17β binding. The maximum [^{3}H]oestradiol-17β binding value was observed after a 2 hr incubation period at 15°C. Similar results were obtained when animals were pretreated with tamoxifen (Fig. 1b).

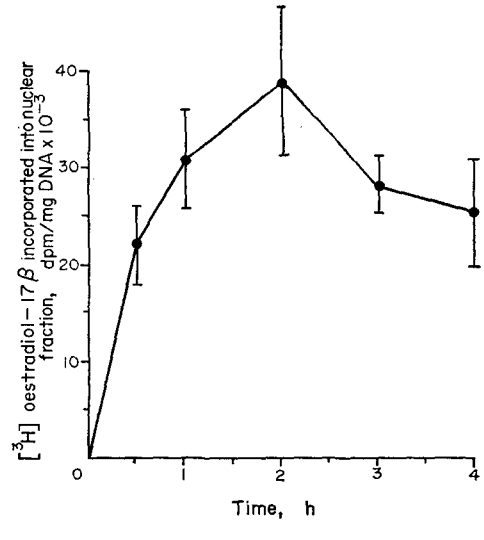

Fig. 2. Nuclear uptake of [^{3}H]*oestradiol-17β. Animals were given* 20 μCi [^{3}H]*oestradiol-17β and tumour biopsy samples removed at time* 0, 0·5, 1, 2, 3, *and* 4 *hr after injection. Nuclear preparations from the biopsies were counted for radioactivity and expressed as dis/min incorporated/mg DNA. Results are the mean ± S.E.M. of five separate tumours.*

Pretreatment of animals with [^{3}H]oestradiol-17β caused the rapid incorporation of radioactivity into mammary tumour nuclear fractions (Fig. 2). The uptake was maximal at 2 hr. Thin layer chromatography of acetone extracts of the nuclear fractions showed the radioactivity

to be associated with oestradiol-17β. Further incubation of the nuclear preparation at 15°C for periods up to 4 hr resulted in a considerable loss of bound radioactivity (Fig. 3). No loss of [^{3}H]oestradiol-17β was observed at 4°C. Inclusion of either oestradiol-17β (20 nmole/l) or tamoxifen (20 nmole/l) in the incubation mixture had no appreciable effect on the amount of radioactivity released.

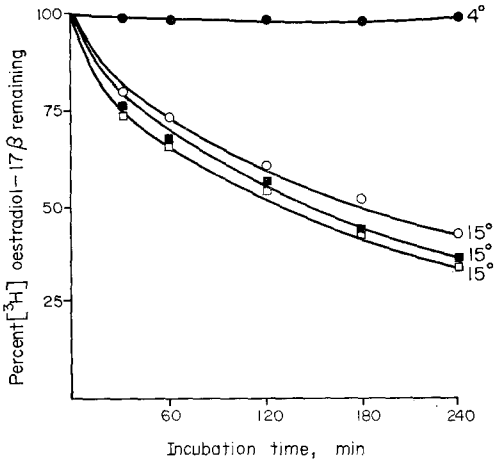

Fig. 3. Rate of exchange of bound [^{3}H]*oestradiol-17β. Animals were given* 20 μCi [3H]*oestradiol-17β 1 hr before sacrifice. Aliquots of the nuclear fraction were treated with protamine sulphate, centrifuged and the precipitated receptor incubated with either Tris buffer,* ●, ○, *oestradiol-17β (20 nmole/l),* ■, *or tamoxifen (20 nmole/l),* □, *at* 4°C *or* 15°C *for periods up to* 4 *hr.*

In view of these results it was considered reasonable that an incubation performed for 2 hr at 15°C would provide an estimate of the oestradiol-17β binding sites present in nuclear fractions of DMBA-induced mammary tumours. Extension of the incubation period or an elevation in the incubation temperature resulted in a nett loss of bound material.

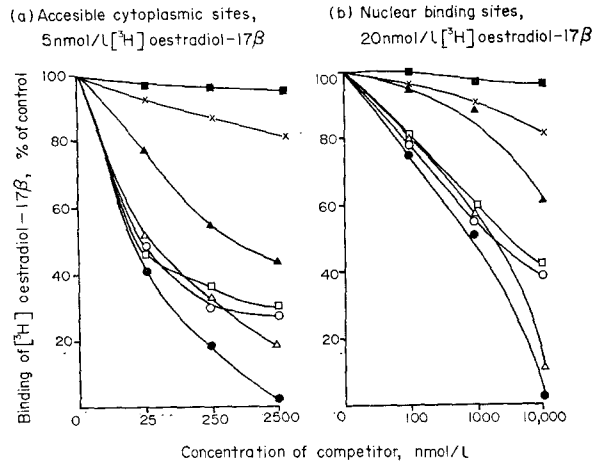

Fig. 5. Specificity of [^{3}H]*oestradiol-17β binding assays.* (a) *Cytosol fractions were incubated for* 16 *hr at* 4°C *with a saturating concentration of* [^{3}H]*oestradiol-17β (5 nmole/l) in the presence and absence of increasing concentrations* (5, 50 *and* 500*-fold excess) of the potential oestrogen antagonists.* (b) *Nuclear fractions were incubated for* 2 *hr at* 15°C *with a saturating concentration of* [^{3}H] *oestradiol-17β (20 nmole/l) under similar conditions to those described in* (a). ●, *oestradiol-17β,* △, *diethylstilboestrol,* ○, *tamoxifen,* □, *CI628,* ▲, meso-*DHBS,* ×, *testosterone and* ■, *corticosterone.*

(b) *Allowance for non-specific oestradiol-17β binding sites.* The binding of oestradiol-17β by non-specific components was a linear function of the [^{3}H]oestradiol-17β concentration (Fig.

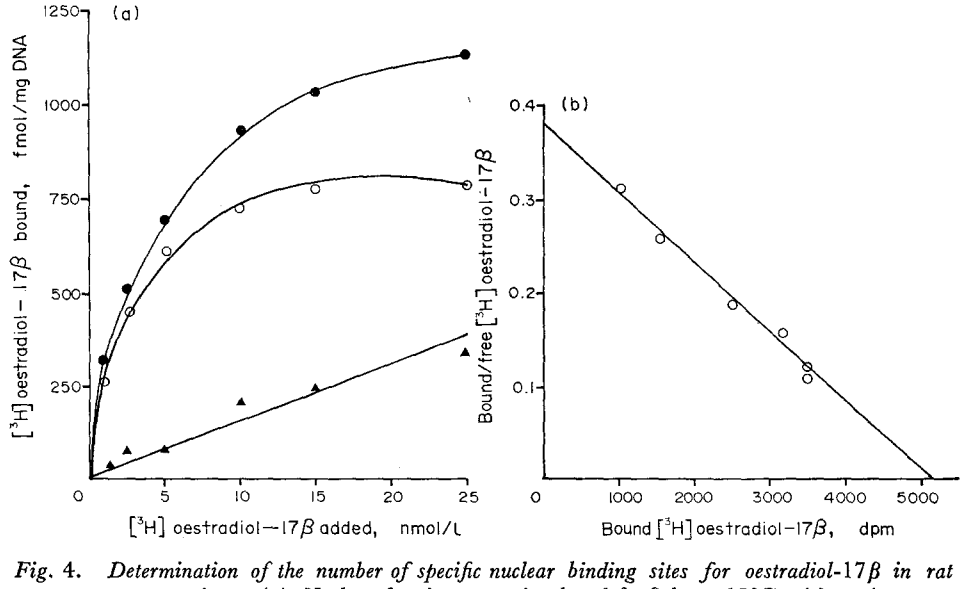

Fig. 4. Determination of the number of specific nuclear binding sites for oestradiol-17β in rat mammary tumour tissue. (a) *Nuclear fractions were incubated for* 2 *hr at* 15°C *with various concentrations of* (1) [^{3}H]*oestradiol-17β (●—●) *or* (2) [^{3}H]*oestradiol-17β plus a* 1000*-fold excess of unlabelled diethylstilboestrol (▲—▲). Specific* [^{3}H]*oestradiol-17β binding was obtained by subtraction of* (2) *from* (1) (○—○), (b) *Scatchard analysis of the corrected values.*

4a). Subtraction of this non-specific binding from the value obtained in the presence of [^{3}H]oestradiol-17β alone, produced a curve demonstrating saturability of binding sites at approximately 15 nmole/l. Analysis of the corrected values by the method of Scatchard [8] gives an apparent dissociation constant, K_D, of 180 pmole/l (Fig. 4b).

Specificity of (a) the accessible cytoplasmic oestradiol-17β binding assay and (b) nuclear oestradiol-17β exchange assay

(a) The affinity of various steroid and non-steroid antioestrogens for the cytoplasmic oestradiol-17β receptor was examined by a competitive binding assay. This involved the incubation of cytosol with a fixed concentration of [^{3}H]oestradiol-17β (5 nmole/l) in the presence and absence of increasing concentrations of the potential oestrogen antagonists (Fig. 5a). Addition of 25 nmole/l oestradiol-17β decreased the binding of [^{3}H]oestradiol-17β to 41% of the control value. The binding continued to decrease with increasing oestradiol-17β concentrations. Testosterone slightly decreased the uptake of [^{3}H]oestradiol-17β whereas corticosterone was without effect. Of the non-steroidal compounds examined diethylstilboestrol, tamoxifen and CI628 were the most effective antagonists.

(b) Competitive binding assays performed on nuclear exchangeable material gave a similar gradation of results (Fig. 5b).

In vivo *effects of oestradiol-17β and tamoxifen*

Within 30 min following *in vivo* administration of 0·5 μg oestradiol-17β a 50% reduction in the binding of [^{3}H]oestradiol-17β to specific cytoplasmic binding proteins was observed (Fig. 6a). The effect was associated with a concomitant progressive increase in specific nuclear oestradiol-17β binding sites. The number of nuclear oestradiol-17β binding sites had increased from a control value of 133 fmole/mg DNA to 369 fmole/mg DNA by 60 min after oestradiol-17β treatment. Elevation of the level of oestradiol-17β (5 μg) administered *in vivo* caused a more pronounced depletion of cytoplasmic binding sites. Nuclear oestradiol-17β binding sites increased to 478 fmole/mg DNA after 60 min. Tamoxifen, like oestradiol-17β, showed similar properties although the response was not as great (Fig. 6b). Injection of saline vehicle had no appreciable effect on either cytoplasmic or nuclear binding sites (Fig. 6c). A secondary period of elevated cytoplasmic

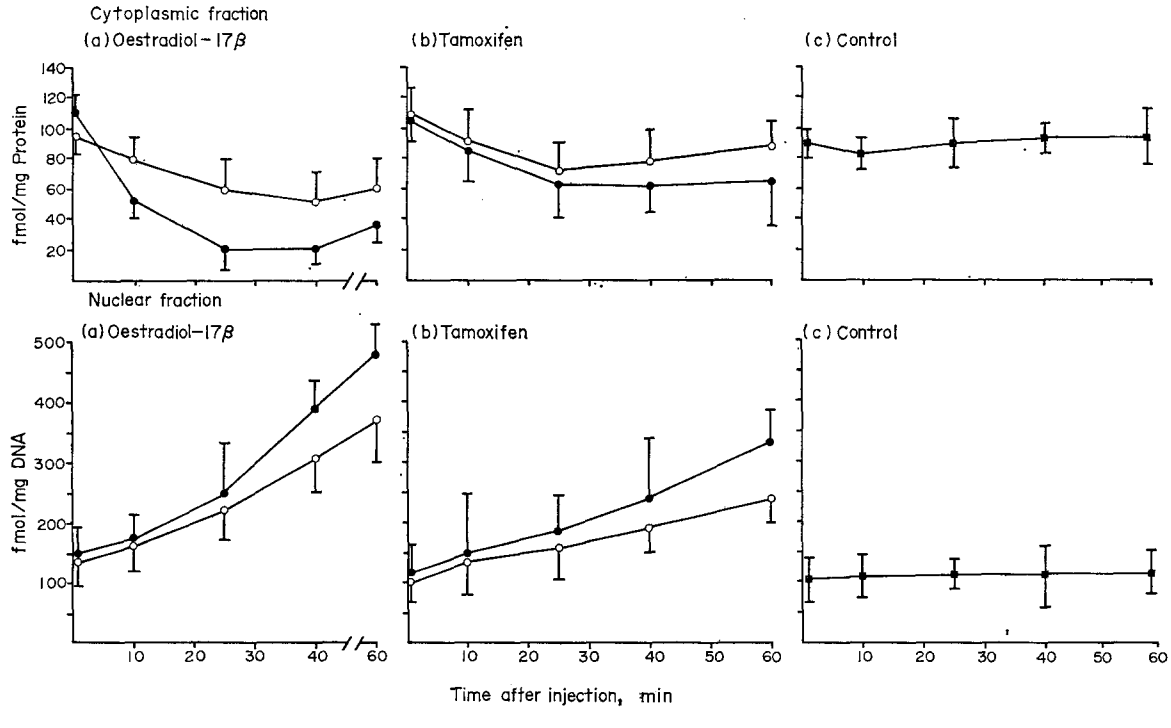

Fig. 6. Effect of in vivo *administration of oestradiol-17β and tamoxifen on cytoplasmic and nuclear oestradiol-17β binding sites. Rats were injected with either* (a) *oestradiol-17β* (0·5 μg, ○ *or* 5 μg, ●), (b) *tamoxifen* (10 μg, ○, *or* 100 μg, ●) *or* (c) *vehicle alone. Tumour biopsy samples were removed at time* 0 *and after* 10, 25, 40 *and* 60 *min. Cytosol preparations from the biopsies were incubated at* 4°C *for* 16 *hr with a saturating concentration of* [^{3}H] *oestradiol-17β* (5·0 *nmole/l*) *or* [^{3}H]*oestradiol-17β plus* 1000*-fold excess* of *stilboestrol. Nuclear preparations were incubated for* 2 *hr at* 15°C *with a saturating concentration of* [^{3}H]*oestradiol-17β* (20 *nmole/l*) *or* [^{3}H]*oestradiol-17β plus* a 1000*-fold excess of stilboestrol. The results are the mean ± S.E.M. of five tumours per group.*

oestradiol-17β binding was observed after either tamoxifen or oestradiol-17β administration (Fig. 7). The results indicate that accessible receptor levels are considerably increased during the 4–24 hr period following oestradiol-17β injection and during the 4–16 hr period following tamoxifen treatment. Tamoxifen, unlike oestradiol-17β, was unable to maintain elevated receptor levels, which showed a considerable decrease in concentration during the 24–48 hr experimental period. Saline injections had no appreciable effect on accessible receptor levels. The concentration of nuclear oestradiol-17β binding sites increased to a

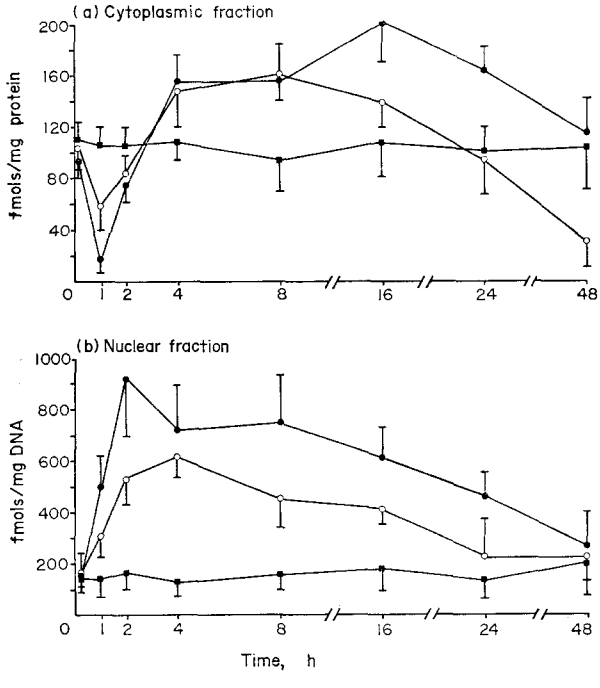

Fig. 7. Effect of in vivo *administration of oestradiol-17β and tamoxifen on cytoplasmic and nuclear oestradiol-17β binding sites. Rats were injected with either* 5 μg *oestradiol-17β* (●); 100 μg *tamoxifen* (○) *or vehicle alone* (■). *The experimental design was as described in the legend to Fig.* 6, *except that tumour tissue was biopsed at time* 0 *and after* 1, 2, 4, 8, 16, 24 *and* 48 *hr.*

maximum (979 fmole/mg DNA) 2 hr after oestradiol-17β administration (Fig. 7) and were still elevated over control values after 24 hr. Administration of tamoxifen produced a less rapid accumulation of nuclear oestradiol-17β binding sites which reached maximum value at 4 hr and returned to pre-stimulation levels at 24 hr.

The administration of tamoxifen (100 μg/day) to tumour-bearing animals on two consecutive days reduced the capacity of the mammary tumour cytosol to bind [^{3}H]-oestradiol-17β (Fig. 8). A further injection of oestradiol-17β (5 μg) at this time did not result in any appreciable nuclear uptake of exchangeable oestradiol-17β or in a stimulation of accessible cytoplasmic oestradiol-17β binding sites. Mammary tumours present in saline treated animals retained their capacity to respond to oestradiol-17β administered *in vivo*.

DISCUSSION

This report describes the development and use of an exchange assay for the determination

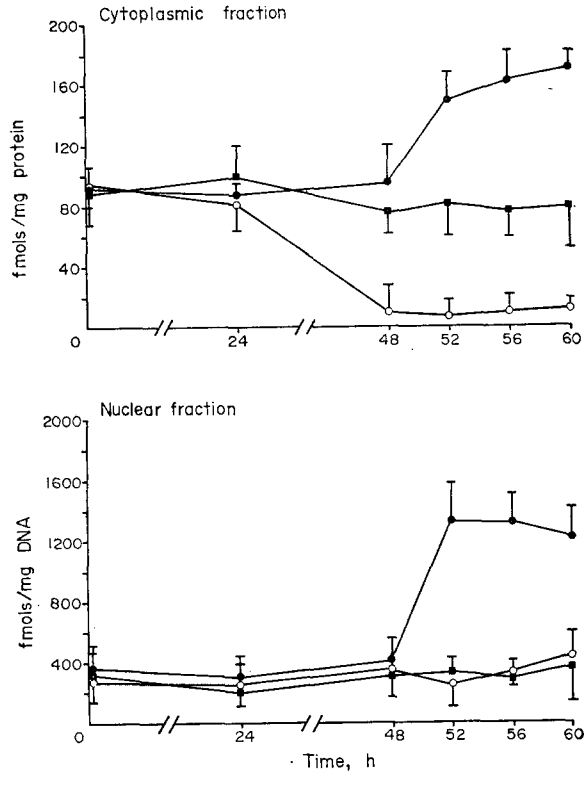

Fig. 8. Effect of tamoxifen pretreatment on the tumour response to oestradiol-17β. Animals were divided into three groups: Group 1 (○) *animals received injections of tamoxifen* (100 μg) *on two consecutive days. After a further* 24 *hr animals were administered oestradiol-17β* (5 μg). *Group* 2 (●) *animals received saline injections for the first two days followed by oestradiol-17β* (5 μg) *on the third. Group* 3 (■) *animals received saline injections alone. Tumour biopsy samples were removed at time* 0, 24, 48, 52, 56 *and* 60 *hr.*

of nuclear oestradiol-17β binding sites present in DMBA-induced mammary adenocarcinomata of rats, irrespective of whether the sites are occupied by oestradiol-17β or tamoxifen. The criteria of minimum loss of binding sites with relatively high rates of exchange were met using an assay temperature of 15°C and an incubation period of 2 hr. The conditions of assay are therefore identical to those described for the estimation of total oestradiol-17β binding sites present in cytoplasmic fractions of DMBA-induced rat mammary tumours [1]. Specificity studies carried out on the binding of

[^{3}H]oestradiol-17β to filled (nuclear) or unfilled (accessible cytoplasmic) binding sites demonstrates that the receptor protein has similar affinity characteristics for non-steroidal oestrogen antagonists in each state. These data suggest a common origin.

The relative ability of the non-steroidal oestrogen antagonists in competing for oestradiol-17β binding sites correlates closely with (a) their relative effectiveness in reducing the *in vitro* capacity of the DMBA-induced mammary tumour to bind [^{3}H]oestradiol-17β following their *in vivo* administration and (b) their ability to cause a regression in tumour mass [2]. It was not possible in that earlier study to determine whether the loss of receptor-binding was due to a decrease of receptor protein or to a strong association of the ligand to the receptor complex, which through steric or transport effects resulted in a biologically inactive complex. The present study demonstrates that tamoxifen can compete with oestradiol-17β for its cytoplasmic receptor protein, although this may be allosteric rather than a direct competition [9]. Other investigations have also demonstrated that tamoxifen can effectively block the association of the oestradiol-17β molecule with its specific receptor protein in DMBA-induced mammary adenocarcinomata [3, 10]. Indeed, we have ascribed an affinity constant of $0{\cdot}213 \times 10^{-2}$l/nmol (cmf $0{\cdot}643 \times 10^{-1}$l/nmol for oestradiol-17$\beta$) to the interaction between tamoxifen and the oestradiol-17β receptor protein [11]. Sedimentation analysis of tamoxifen treated cytosol fractions [3, 10] demonstrates that the anti-oestrogen competes for 8S-oestrogen binding components.

Although the initial association of tamoxifen with its specific cytoplasmic binding site is undoubtedly important, this present study indicates that it forms only part of the complex mechanism by which tamoxifen influences tumour growth. After the *in vivo* injection of tamoxifen or oestradiol-17β, a rapid dose-dependent accumulation of nuclear [^{3}H]-oestradiol-17β exchangeable material occurs. Maximum [^{3}H]oestradiol-17β binding values were observed 2 hr following the administration of oestradiol-17β and 4 hr after tamoxifen treatment. Oestradiol-17β more effectively promoted the translocation process than did tamoxifen, which may reflect either their relative rates of entry into the tumour cells [1, 10, 12], or their different affinities for the oestradiol-17β receptor protein.

The translocation of the receptor complex to the nucleus results in an initial depletion of both total [1] and accessible cytoplasmic oestradiol-17β binding sites. Tamoxifen is able to elicit further oestrogen-like actions which result in elevated cytoplasmic binding site levels. The above data are not compatible with the hypothesis that the sole action of tamoxifen lies in its ability to compete with oestradiol-17β for its cytoplasmic binding sites. It would appear that tamoxifen, bound to the cytoplasmic binding protein, enters the nucleus and associates with chromatin acceptor sites, thus affecting the rates of transcription of the DNA template. Such a phenomenon has been demonstrated in other studies from this group [13] which show that the administration *in vivo* of either oestradiol-17β or tamoxifen brings about an early elevation in nuclear RNA polymerase B activity. It would seem reasonable that the secondary effects observed in the cytoplasmic oestradiol-17β receptor levels following tamoxifen treatment are a direct consequence of an interaction of the tamoxifen-receptor complex with chromatin, resulting in the production of mRNA species coding for specific proteins including the receptor for oestradiol-17β.

Tamoxifen, unlike oestradiol-17β, is unable to maintain elevated cytoplasmic receptor levels, which diminish during the 24–48 hr experimental period. The effect was also reported by Clark, Anderson and Peck [14] and Rochefort and Capony [15] for nafoxidine and for CI628 by Katzenellenbogen and Furguson [16] using rat uteri. The results of Clark *et al.* [14] clearly indicate that nafoxidine acts as an atypical oestrogen in that it can stimulate uterine growth over extended periods of time (up to 19 days), but when administered either simultaneously with oestradiol-17β or when injected 24 hr before oestradiol-17β, acts as an oestrogen-antagonist. Furthermore, the nafoxidine-receptor complex present in nuclear fractions remains elevated, although there is no apparent replenishment of the cytoplasmic oestradiol-17β binding protein. The elevated retention of the ligand-receptor complex was not noted in DMBA-tumour nuclear fractions following the administration of tamoxifen. This may in part account for its anti-tumour activity since continued high levels of ligand-receptor complex seem to be equatable with oestrogen-like responses. However, the small amounts of [^{3}H]oestradiol-17β exchangeable material present in rat mammary tumour nuclei 24 hr and 48 hr after tamoxifen treatment should not be disregarded. It may represent a limited number of tamoxifen–receptor complexes in chromatin resulting from

either a relatively strong association of the receptor with specific acceptor sites or it may be a consequence of a low clearance rate for tamoxifen and its metabolites from the animal [17].

Tamoxifen treatment therefore results in a depletion of tumour cytoplasmic oestradiol-17β binding sites, a condition which appears to be refractory to further oestrogen action, since *in vivo* administration of oestradiol-17β at this time fails to stimulate receptor uptake into tumour cells nuclei or replenish cytoplasmic binding sites. The tumour is therefore in an oestradiol-17β non-responsive state, a condition which must ultimately result in a regression of tumour mass.

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Incidence Risk Factors and Survival in Breast Cancer: Report on Five Years of Follow-up Observation*

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Abstract—*In a previous report three year survival rates in breast cancer were related to geography, socio-economic status and age at first pregnancy. Here, results from 5 years of follow-up observations are summarized with respect to incidence risk factors. Age-adjusted 5 year survival rates were 57·3% in Boston, Massachusetts, 49·5% in Glamorgan, Wales, 41·9% in Slovenia, Yugoslavia, and 74·9% in Tokyo, Japan; corresponding age-adjusted annual incidence rates per 100,000 were 55·0, 38·8, 24·4 and 12·9. Although survival rates varied markedly among these areas, there was not a consistent relationship between incidence rate and probability of survival. Differences in survival rates between Boston, Glamorgan and Slovenia were reduced when examined according to extent of disease at diagnosis, but the relatively high survival rate in Tokyo was not explained. Survival rates tended to increase, although inconsistently, with increasing socioeconomic status as measured by duration of schooling. Probability of survival was not related to childbearing or, for parous patients, to age at first full-term birth.*

THREE year survival rates of female breast cancer patients in Boston, Massachusetts, Glamorgan, Wales, and Tokyo, Japan, were related previously to incidence rate of the disease, and to duration of schooling and age at first pregnancy, two variables which are associated with risk of developing the disease [1]. The survival rate was highest in Tokyo, where the incidence rate is very low. However, the lowest survival rate was observed in Glamorgan, where the incidence rate is lower than in Boston, but much higher than in Tokyo. Differences in survival rates between these areas were not explained by differences in the extent of disease at diagnosis. The probability of survival was only inconsistently related to duration of schooling, and was unrelated both to childbearing and to the age at which parous patients first became pregnant. The purposes of the present report are: (1) to present survival data from five years of observation in these three areas and in an additional area, Slovenia, Yugoslavia, and (2) to summarize available evidence on the relationship of incidence factors to survival.

MATERIAL AND METHODS

The series of patients described here were assembled during a collaborative international study of etiologic factors in female breast cancer [2]. In Boston, Glamorgan and Slovenia nearly all newly diagnosed cases of breast cancer in geographically defined populations were ascertained during the period of observation. Ascertainment was not complete in Tokyo, but the cases are believed to be about half of those in the prefecture of Tokyo-to, and to be representative of new cases in the area. The characteristics of the patient series have been reported [3–6].

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The extent of tumor at diagnosis was assessed from information in the record of the initial hospitalization for breast cancer. Only information obtained before biopsy or treatment was used for the clinical assessment. For pathologic assessment, a patient was required to have had regional lymph nodes examined histologically or to have had a histologically confirmed metastasis. Otherwise, the pathologic extent of disease was considered "unknown". A tumour was classified as "localized" if there was no evidence of extension beyond the breast. Evidence of spread no further than to ipsilateral skin, chest wall or axillary or subclavicular lymph nodes was considered "regional". Greater extent was designated as "distant" disease.

The original patient series numbered 758 in Boston, 710 in Glamorgan, 831 in Slovenia and 861 in Tokyo. Five cases in Boston, two in Glamorgan, one in Slovenia and six in Yokyo subsequently were found not to be eligible index cases and were excluded. Survival was measured from the date of the first hospitalization during which a diagnosis of breast cancer was made. The proportions of eligible patients successfully followed to five years were high—98·9% in Boston, 97·9% in Glamorgan, 99·4% in Slovenia and 98·5% in Tokyo. Therefore, the present report is based on the successfully followed patients only, without consideration of patients lost to follow-up.

Survival rates have been adjusted for age by the direct method of standardization [7] and these rates are accompanied by their standard errors. In the tables, numbers in parentheses are the numbers on which the survival rates are based. Rates are not given for categories with less than 25 patients.

RESULTS

Table 1 gives adjusted rates of survival to the end of each year of observation, crude five-year survival rates, and age-adjusted incidence rates in each area. After each year of observation, the survival rate was highest in Tokyo, followed, in descending order, by the rates in Boston, Glamorgan and Slovenia. Among these areas, the disparity in survival rates is considerable; the five year survival rate in Tokyo is 1·8 times the rate in Slovenia. The relationship of survival rate to incidence rate is irregular: the highest survival rate, in Tokyo, is associated with the lowest incidence rate but the lowest survival rate, that in Slovenia, was observed in the area with the next lowest incidence rate.

Distributions of patients according to clinical extent of disease at diagnosis are presented in Table 2. The highest percentage of patients with localized disease was observed in Boston and the lowest percentage in Slovenia. Patients in Tokyo had the lowest percentage of distant disease. As with survival rates, there is no simple relationship between incidence risk and extent of disease. Comparisons of pathologic extent are not presented since these distributions could be affected very greatly by therapeutic practices. In Slovenia, for example, where non-surgical management of breast cancer is relatively frequent [8], pathologic extent was "unknown" for 49·2% of patients.

Survival rates according to clinical extent of disease are presented in Table 3. Data for each year of follow-up are given for local and regional disease, where the numbers of patients were greatest. Except for the few patients with unknown extent, the high survival rate of the Japanese patients was evident in each extent category and after each year of follow-up observation. Differences in survival rate between Boston, Glamorgan and Slovenia were small among patients with localized tumors. For patients in these areas who had tumors classified as regional, the survival rate after each year of follow-up was highest in Boston, as was the rate in the total group of patients. After one year, the survival rate was slightly higher in Slovenia than in Glamorgan. In successive years, the survival rate was higher in Glamorgan, the greatest difference between these two areas occurring after three years.

Five year survival rates according to pathologic extent are given in Table 4. Again, the favorable survival experience of the Japanese patients is observed consistently. Extent-specific differences in survival rates between Boston, Glamorgan and Slovenia tended to be smaller than the differences in the overall rates, but the large numbers of patients with unknown pathologic extent, particularly in Glamorgan and Slovenia, make interpretation of these results difficult.

Five year survival rates are shown by duration of schooling in Table 5. Generally, the rates tended to rise with increasing duration of schooling. In Tokyo the increase in survival was small compared to that in the other centers. Although there was a marked rise in survival rate from the first to the second level of education in Slovenia, the survival rate of patients who had 12 to 15 years of schooling was again quite low.

Survival rates also were examined in relation to childbearing and to the age at first full-term

Table 1. Cumulative age-adjusted rates of survival to end of each year of follow-up, crude five year survival rates, and age-adjusted incidence rates

Year	Center			
	Boston	Glamorgan	Slovenia	Tokyo
1	90·0 ± 1·1	87·0 ± 1·3	83·0 ± 1·3	94·3 ± 1·0
2	79·1 ± 1·6	73·9 ± 1·7	64·9 ± 1·7	88·2 ± 1·3
3	69·4 ± 1·8	63·4 ± 1·8	53·8 ± 1·7	82·2 ± 1·5
4	62·0 ± 1·9	55·8 ± 1·9	48·0 ± 1·7	78·3 ± 1·6
5	57·3 ± 1·9	49·5 ± 1·9	41·9 ± 1·7	74·9 ± 1·7
Crude five year survival rate	56·0 ± 1·8	48·6 ± 1·9	41·9 ± 1·7	76·0 ± 1·5
Number of patients	745	693	825	842
Age-adjusted annual incidence per 100,000	55·0	38·8	24·4	12·9

Table 2. Percentage of distributions of extent of disease assessed by clinical criteria

		Center			
		Boston	Glamorgan	Slovenia	Tokyo
Total number of cases		745	693	825	842
Percent extent unknown		10·5	5·2	5·0	3·2
Number with known extent		667	657	784	815
Percent	Local	52·5	41·2	23·6	43·8
	Regional	37·4	46·6	62·6	50·9
	Distant	10·0	12·2	13·8	5·3
	Total	99·9	100·0	100·0	100·0

Table 3. Cumulative age-adjusted survival rates according to clinical extent of disease

Clinical extent	Year	Center			
		Boston	Glamorgan	Slovenia	Tokyo
Local	1	94·6 ± 1·2	96·6 ± 1·1	95·2 ± 1·6	97·5 ± 1·4
	2	89·0 ± 1·7	86·4 ± 2·1	86·9 ± 2·5	93·8 ± 1·8
	3	79·5 ± 2·3	76·6 ± 2·6	79·8 ± 3·0	91·3 ± 2·1
	4	71·4 ± 2·5	69·6 ± 2·8	73·7 ± 3·3	89·5 ± 2·2
	5	66·7 ± 2·6	66·9 ± 2·8	68·0 ± 3·5	86·0 ± 2·4
		(350)	(271)	(185)	(357)
Regional	1	92·3 ± 1·7	84·8 ± 2·0	86·2 ± 1·5	94·8 ± 1·2
	2	75·2 ± 3·0	70·4 ± 2·6	65·4 ± 2·1	87·7 ± 1·8
	3	65·8 ± 3·3	61·0 ± 2·8	52·4 ± 2·3	80·3 ± 2·1
	4	58·6 ± 3·4	51·6 ± 2·9	46·6 ± 2·2	74·8 ± 2·3
	5	52·7 ± 3·4	42·2 ± 2·9	39·9 ± 2·2	71·4 ± 2·4
		(250)	(306)	(491)	(415)
Distant	5	11·5 ± 3·5	15·1 ± 4·5	5·6 ± 2·4	20·0 ± 4·9
		(67)	(80)	(108)	(43)
Unknown	5	67·4 ± 5·5	52·5 ± 8·1	50·1 ± 7·8	66·4 ± 8·4
		(78)	(36)	(41)	(27)
Total	5	57·3 ± 1·9	49·5 ± 1·9	41·9 ± 1·7	74·9 ± 1·7
		(745)	(693)	(825)	(842)

Table 4. Five year age-adjusted survival rates according to pathologic extent of disease

Center	Pathologic extent				Total
	Local	Regional	Distant	Unknown	
Boston	79·8 ± 2·5 (291)	51·2 ± 3·0 (293)	— (10)	17·5 ± 3·9 (151)	57·3 ± 1·9 (745)
Glamorgan	71·6 ± 3·4 (176)	46·7 ± 3·0 (268)	— (13)	34·7 ± 3·5 (236)	49·5 ± 1·9 (693)
Slovenia	78·1 ± 3·2 (179)	47·7 ± 3·5 (225)	— (15)	23·1 ± 2·1 (406)	41·9 ± 1·7 (825)
Tokyo	89·7 ± 1·9 (382)	63·9 ± 3·0 (354)	33·9 ± 8·3 (46)	68·0 ± 5·8 (60)	74·9 ± 1·7 (842)

*Table 5. Five year age-adjusted survival rates according to duration of schooling of patient**

Center	Duration of schooling (years)				Total
	< 8	8–11	12–15	16+	
Boston	52·2 ± 8·0 (50)	53·1 ± 4·5 (166)	63·1 ± 3·0 (280)	69·0 ± 5·6 (67)	60·3 ± 2·2 (563)
Glamorgan	— (23)	51·6 ± 2·2 (509)	66·9 ± 6·8 (52)	— (15)	51·9 ± 2·0 (599)
Slovenia	39·1 ± 2·2 (491)	60·2 ± 3·9 (176)	41·2 ± 6·7 (61)	— (7)	44·3 ± 1·8 (735)
Tokyo	72·2 ± 4·1 (142)	75·0 ± 2·3 (529)	80·2 ± 3·9 (143)	— (9)	75·1 ± 1·7 (823)

*In this table and in Table 6, patients were excluded who were not interviewed or whose interview was rated "unreliable".

Table 6. Five year age-adjusted survival rates according to age at first full-term birth. Married women only

Center	Nulliparous	Parous, age at first birth being			Total
		< 25	25–29	≧ 30	
Boston	69·0 ± 5·0 (91)	62·6 ± 4·4 (129)	55·1 ± 4·5 (125)	63·0 ± 4·6 (108)	61·2 ± 2·4 (463)
Glamorgan	52·7 ± 5·1 (99)	51·9 ± 3·6 (187)	50·6 ± 4·2 (148)	55·6 ± 4·8 (108)	52·5 ± 2·1 (542)
Slovenia	35·0 ± 6·0 (63)	43·8 ± 3·2 (236)	44·1 ± 3·5 (202)	42·6 ± 4·0 (139)	43·7 ± 2·0 (640)
Tokyo	78·5 ± 3·6 (154)	72·6 ± 2·9 (268)	76·5 ± 3·1 (229)	74·9 ± 5·2 (111)	74·9 ± 1·8 (762)

birth of parous women (Table 6). Probability of survival to five years following diagnosis was neither strongly nor regularly related to either of these characteristics.

DISCUSSION

Principally because of the relatively high survival rate in Japan, where the incidence rate is very low, consideration has been given to the possibility that the survival rate of breast cancer patients is inversely related to the incidence rate of the source population [1, 9, 10]. The observation that breast cancer in Japan appears less aggressive, histologically, than does breast cancer in the United States [11, 12] has tended to support this idea.

Accumulating data, however, strongly suggest that survival does not have a simple relationship to incidence risk. (A) Although the survival rate of patients in Japan is high, the relationship between incidence and survival is

not regular. For instance, the survival rate in the United States consistently has been higher than that in England [9], where the incidence rate is lower than in the U.S. (B) Although duration of schooling is related, albeit somewhat irregularly, to survival rate, the patients with most education, representative of those at highest incidence risk on this variable, have the highest survival rates. The basic geographic observation, high survival rates in Japan where risk is low, is in the opposite direction. (C) Age at first birth, a stronger risk factor than duration of schooling, has appeared unrelated to survival. (D) Removal of the ovaries before age 35 is associated with a substantial reduction in breast cancer risk [13, 14]. The probability of survival of breast cancer patients was observed to be related to pelvic surgery before development of the malignancy [15, 16]. However, the survival advantage associated with prior pelvic surgery did not appear to depend on removal of the ovaries nor on whether the operation was performed before natural menopause [16]. (E) International differences in histology have not explained the high survival rate in Japan despite the relatively frequent occurrence of favorable histologic characteristics among breast cancer patients there [10–12, 17].

In conclusion, the major known indicators of breast cancer risk do not appear to be important determinants of the survival of patients affected with the disease. Because breast cancer is a commonly occurring malignancy, and because the overall outlook for patients with this disease is not favorable, investigation of the natural history of breast cancer should continue.

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Studies of the Effects of Busulphan on the Regeneration of Rat Liver

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Abstract—*Regeneration of rat liver is retarded by busulphan (4·4 mg/kg of body weight) given interperitoneally at the time of partial hepatectomy. The incorporation of tritiated thymidine into liver DNA is diminished in busulphan-treated animals and it is suggested that the drug prevents cells from entering the proliferative stage. Busulphan is without effect on the duration of the mitotic phase of the cell cycle of the parenchyma, but causes a slight prolongation of the durations of the S and G_2 phases and a partial inhibition of the passage of cells from the G_2 to the M phase of the cycle.*

INTRODUCTION

THE WIDE use of busulphan (1,4-dimethanesulphonyloxybutane, "Myleran") and its derivatives in the treatment of leukaemia stems from their selectivity of action on haemopoietic tissues [1]. The primary site of action of busulphan and other alkylating agents is considered by some authors [2–4] to be the mitotic apparatus. Busulphan presents many of the features of a monofunctional alkylating agent toward DNA [5, 6; but see 2] and its action has been attributed to depurination and consequent single-strand breakage of the nucleic acid [5]. It is well documented, however, that busulphan has effects other than on the mitotic apparatus [4, 7] and part of the injury to the chromosomes could arise indirectly from lysosomal damage [8]; the drug binds not only to DNA but also to RNA and protein [2]. The multiplicity of possible reactions of alkylating agents with tissue components makes it difficult to designate any single reaction as the cause of cytotoxicity [7, 9].

There are conflicting reports concerning the phase(s) in the cell cycle at which busulphan acts. It has been concluded that busulphan and "Myleran-type" drugs arrest bone marrow "stem cells" in the resting, G_0 phase of the cell cycle rendering them unable to produce a normal differentiated population [10]. In rat epithelium, however, there is evidence that the drug acts in the G_1 phase of the cell cycle in a manner that allows cells to initiate and complete DNA synthesis normally but that prevents their subsequent mitotic division [11].

In the use of chemotherapeutic agents it is desirable to know what consequences these have on cells of normal tissues and on the ability of such cells to respond to proliferative stimulus subsequent to repair or regeneration. Busulphan is known to cause marked cellular changes in organs other than bone marrow during the treatment of patients with chronic myelogenous leukaemia [12]. The effects of a single dose of busulphan on a rapidly proliferating tissue, regenerating liver, are presented here, together with studies into the possible points of action of the drug in the cell cycle of the parenchyma.

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MATERIAL AND METHODS

Animals

Four to six-week old male rats, *Mus norwegicus* of the hooded Lister strain, random bred and weighing between 80–150 g were used in the studies.

Partial hepatectomy and drug administration

Partial hepatectomy of *ca.* 70·2% (determined by direct measurement on 10 rats) of the liver, consisting of removal of the median and left lateral lobes, was performed under ether anaesthesia by the method of Higgins and Anderson [13]. Operations were carried out between 6 a.m. and noon to minimise diurnal variation in regenerative ability [14]. Animals were allowed food (Oxoid diet 41B; Oxoid, London, Eng.) and 5% dextrose *ad libitum*

following operation and were maintained at 22°C.

Busulphan (1,4-dimethanesulphonyloxybutane) was administered (4·4 mg/kg of body weight, in an emulsion of dimethyl sulphoxide–arachis oil, (9/1, v/v) [10]. Doses (0·5 ml) of busulphan suspension or, for control animals, drug-free dimethylsulphoxide–arachis oil emulsion were given intraperitoneally before completion of suturing.

Histological preparation and autoradiography

Animals were sacrificed by heavy ether anesthesia and cervical dislocation. Histological sections were prepared from portions of the right laterial lobe of livers and examined by high resolution autoradiography using the liquid emulsion-dipping method, as described by Fabrikant [15]; slides were stained with haematoxylin and eosin after autoradiography. Parenchymal mitoses were scored as labelled if an arbitrary lower limit of 5 grains per mitotic figure was exceeded.

Estimation of nucleic acids

The contents of RNA and DNA in liver samples were determined by a modification of the Schmidt–Thannhauser method [16], estimating RNA spectrophotometrically [17] and DNA using an improved diphenylamine method [18]. The phosphorus content of calf thymus DNA (BDH Chemicals Ltd., Poole, Eng.), used as DNA standard, was determined by the method of Allen [19].

Incorporation of tritiated thymidine (TdR-6 ^{3}H) into DNA

Partially hepatectomized rats were injected at 6 hr intervals with TdR-6 ^{3}H (specific activity 20–30 Ci/mM, purchased from Radiochemical Centre, Amersham, England; 0·5 μCi/g of body weight in 0·5 ml normal saline given intraperitoneally) and sacrificed after 45 min. Assays of radioactivity in DNA extracts, prepared as above, were carried out with a Packard tri-Carb liquid scintillation counter (Packard Instruments Co., Ill., USA) using the emulsion technique (tT21) of Patterson and Green [20]. DNA samples (1 ml in 1N $HClO_4$ with 10 ml scintillant) were counted with an efficiency of *ca.* 27%. A quench curve was prepared by addition of 8–10 μl of nitromethane to vials containing 10 ml scintillant and 1 ml N $HClO_4$ containing a known amount of TdR-6 ^{3}H.

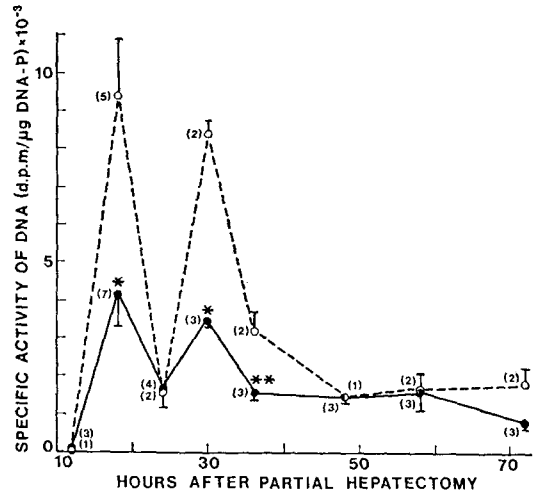

Fig. 1. Incorporation of tritiated thymidine into DNA of regenerating rat liver.

Legend: The mean values for control (○) and busulphan-treated animals (●) are shown. The number of rats employed are given in brackets; 4 analyses were made on each of two duplicate samples of liver from each rat. The vertical lines illustrate S.E.M. Values which differ significantly from those of controls ($P < 0·05$ and $P < 0·1$**) are indicated.*

Duration of cell cycle phases

Rats were injected intraperitoneally with TdR-6 ^{3}H (0.5 μCi/g of body weight in 0.5 ml normal saline) 16 hrs after partial hepatectomy, killed at hourly intervals, and the fraction of labelled (5 grains or more) parenchymal mitoses counted. A minimum of a hundred mitoses

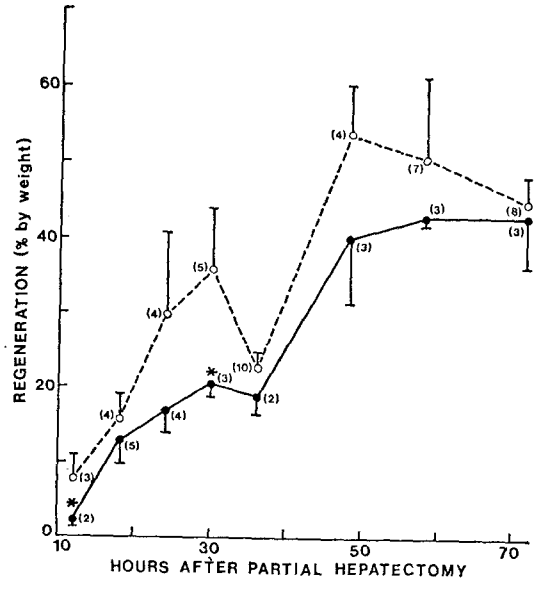

Fig. 2. Restoration of liver mass following partial hepatectomy.

Legend: The mean value for control (○) and busulphan-treated animals (●) are shown. The number of rats used are given in brackets. The vertical lines represent S.E.M. Values which differ significantly from those of controls ($P < 0·1$) are indicated.*

were scored for each rat and the phases of the mitoses (pro-, meta-, ana- or telo-phase) were recorded. The proportion of abnormal mitotic figures in each phase was noted, taking the criteria of abnormality given by Horst and Rudnicki [21]. The durations of the cell cycle phases (T_{G_1}, T_S, T_{G_2} and T_M) were determined by analyses of percentage labelled mitoses curves [22]. The means and standard deviations of T_{G_2} and T_S were derived from probit plots of the faction of labelled mitoses [23]. An approximate value for the duration of the complete cell cycle (T_C) for control rats was estimated from corresponding points on the first and second waves of labelled mitoses. The durations of G_2 (minimum) + M/2 were taken as the distance on the abscissae from the times of injection of tritiated thymidine to the mid-points of the ascending limbs of the waves of labelled mitoses. Prophase durations were derived from the differences of the times of interception on the abscissa of the prophase and metaphase labelling curves or from the half-maximal labelling intercepts of the ascending limbs of these curves; the times of the other mitotic phases, and thus of T_M, were calculated from the percent of mitoses in each phase over the period (19–28 hr after operation) of study [24]. The approximate duration of the G_1 phase of the cell cycle (T_{G_1}) was derived from T_C and the length of the other cell cycle phases.

Mitotic indices curves were plotted with values corrected for the increasing mass of liver [15].

RESULTS

The effect of busulphan on the incorporation of TdR (6-^{3}H) into DNA of regenerating rat liver is shown in Fig. 4. The rate of DNA synthesis is considerably reduced during the first 48 hr of regeneration following treatment with the drug and the specific activity of DNA is lowered by *ca.* 60% at the peaks (18 and 30 hr) of synthesis. The restoration of liver weight is, arguably, retarded by busulphan during this time interval (Fig. 2); the DNA and RNA contents of the regenerating tissue, however, are not significantly changed by the drug. (Fig. 3a, b).

The percentage labelled mitoses curve of the parenchymal cells of busulphan-treated rats, obtained following pulse-labelling with TdR-^{3}H, is displaced on the time axis by *ca.* 1 hr (Fig. 4). The mean durations of the DNA synthetic period, or S phase of the cell cycle (T_S), and of the premitotic G_2 phase (T_{G_2}), estimated by probit analyses of the curves, are slightly prolonged following drug-treatment (Table 1) without any appreciable change in the spread of the durations (cf. coefficients of variance); an increase in the minimum duration of the G_2 phase is also found. The single dose of busulphan does not alter the duration of the mitotic phase (T_M) since the time taken for the ascending limb of the curve to rise to maximal labelling ($\approx T_M$) remains unaltered.

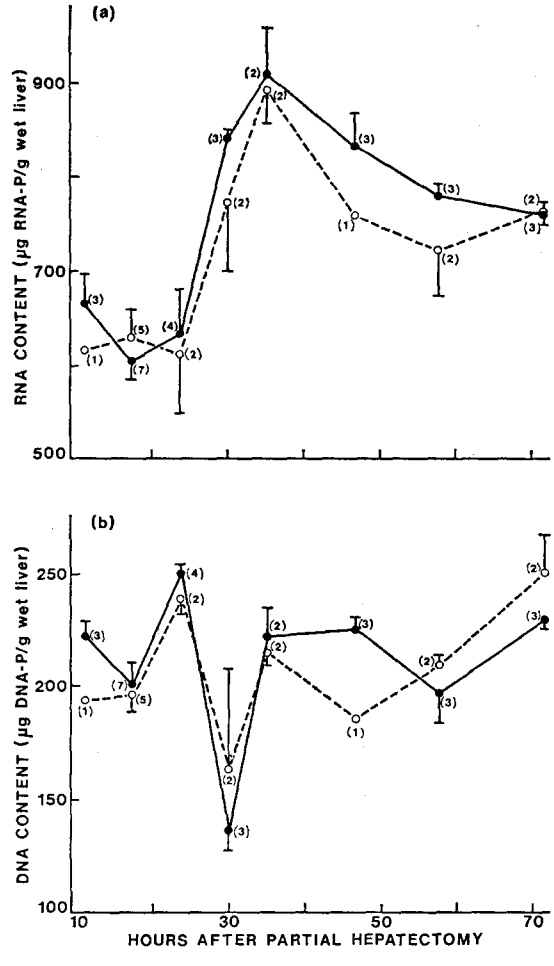

Fig. 3. Effect of busulphan on (a) the RNA content and (b) the DNA content of regenerating rat liver.

Legend: The mean values for control (○) and busulphan-treated animals (●) are shown. The number of rats used are given in brackets; 4 analyses of DNA were made on each of two duplicate samples of liver from each rat; one determination of RNA content was made for each rat. The vertical lines represent S.E.M. Experimental values do not differ significantly from control values ($P > 0{\cdot}1$).

Similarly, a study of curves of labelled mitoses for the individual mitotic phases (Fig. 5), which are displaced by *ca.* 1 hr for busulphan-treated rats, provides no evidence that the drug causes any significant alteration in the durations of phases or in the total time of mitosis (Table 2); a partial blockage in any of the mitotic phases would have led to a change in

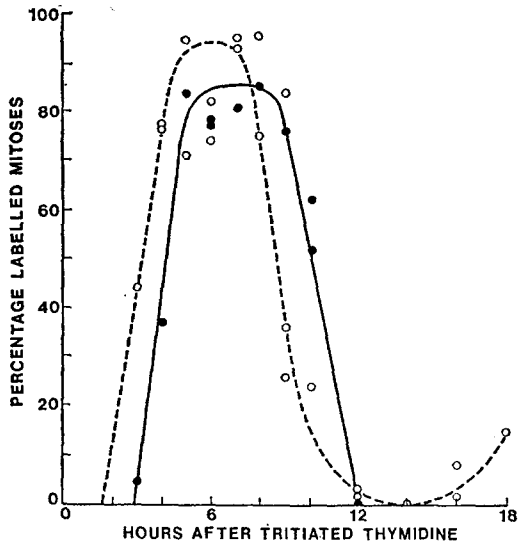

Fig. 4. Percentage labelled mitoses of parenchymal cells of partially hepatectomised rats recorded at intervals after pulse-labelling with tritiated thumidine.

Legend: ○, controls; ●, animals treated with busulphan. Both groups were injected with tritiated thymidine at 16 hr after operation, which corresponds to the origin (0 hr) on the abscissa. Each circle represents the value for one rat; 100 mitoses were scored for each animal.

Table 1. Duration of the cell cycle phases of parenchyma in regenerating rat liver

Phase		Duration (T) (hr)	Standard deviation (σ) (hr)	Coefficient of variance (σ/T)
G_1	C	~ 2·2	—	—
	B	—	—	—
S	C	5·5[a]	1·2	0·22
	B	6·0[a]	1·4	0·23
G_2	C	3·4[a]	1·2	0·35
		1·1[b]	—	—
	B	4·2[a]	0·7	0·17
		2·1[b]	—	—
M[c]	C	1·9	—	—
	B	2·0	—	—
T_C[d]	C	~ 13·0	—	—
	B	—	—	—

Legend: C, control animals. B, animals treated with busulphan. [a]mean duration. [b]Minimum duration, obtained from values of G_2 (minimum) + (M/2) and T_M. [c]T_M derived from sum of individual mitotic phases (Table 2). [d]Total cell cycle time.

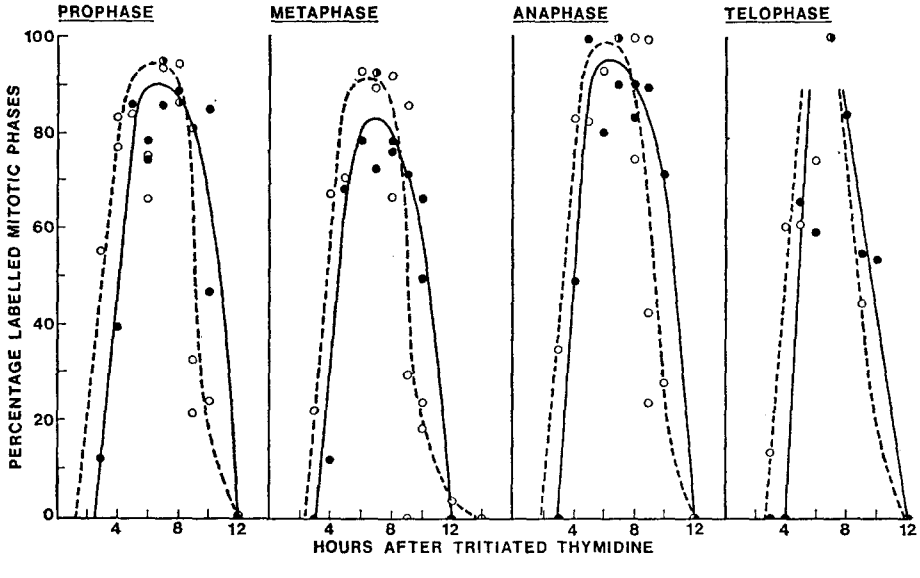

Fig. 5. Percentage labelled mitotic phases of parenchymal cells of partially hepatectomised rats recorded at intervals after pulse labelling with tritiated thymidine.

Legend: ○, controls; ●, animals treated with busulphan. Both groups were injected with tritiated thymidine at 16 hr after partial hepatectomy, which corresponds to the origin (0 hr) on the abscissa. Each circle represents the number of mitotic phases in 100 mitoses scored for one rat.

the estimate of duration of the phase and subsequent phases. Lower maximal values of percentage labelled mitoses (and mitotic phases) are reached, however, following administration of the drug.

It was not possible to calculate the total cell cycle time (T_C) for busulphan-treated animals as they showed only one wave of mitotic labelling (Fig. 4).

An increase (< 15% of the total number of mitoses) was observed in the level of abnormal mitotic figures for drug-treated animals be-

Table 2. Durations of the individual mitotic phases of parenchyma in regenerating rat liver

Phase	Duration (hr)	
	Control rats	Busulphan-treated rats
Prophase	1·0[a]	1·1[b]
Metaphase	0·72	0·65
Anaphase	0·16	0·18
Telophase	0·05	0·09

Legend: [a]Derived from half-maximal labelling intercepts of ascending limbs of prophase and metaphase labelling curves. [b]Obtained from differences of times of interception on abscissa of prophase and metaphase labelling curves.

tween 16 and 30 hr after partial hepatectomy; the abnormalities were not restricted to any particular phase of mitosis.

The parenchyma of control animals showed a major peak of mitotic activity (mitotic index, 5%) at 26 hr and a minor peak of activity (mitotic index, 2%) at 22 hr following operation. Measurements of mitotic index for drug-treated animals were too variable to ascribe to the drug an effect on mitotic activity.

DISCUSSION

The dose of busulphan used in the present study was chosen so as to be less than the total amount (mg/kg body weight) administered over a period of several weeks, to achieve remission status, to patients with chronic myelocytic leukaemia [25]; it is well below the lethal level for rats [1]. The long half-life of the drug (11–12 hr [26]) ensures that it is viable, when administered interperitioneally immediately following partial hepatectomy, throughout the first cycle of the stimulated proliferative growth of the parenchymal cells; young rats show two peaks of DNA synthesis, in contrast to older animals [14].

The single dose of busulphan caused a small increase in the level of parenchymal mitotic abnormalities, but these were not restricted to a particular mitotic phase [11]; continued administration of busulphan is known to cause injury to the mitotic apparatus [3].

The relatively minor effects of busulphan on the S and G_2 phases of proliferating parenchymal cells and its lack of action on the durations of the mitotic phases is consistent with the insensitivity of the later phases of the cell cycle of "stem cells" to the drug [10].

The reduced level of labelled mitoses (and mitotic phases) following administration of the drug can not be attributed to increased spread in the duration of the G_2 phase. It may be explained [27] by an increased flux of cells from a higher-order polyploid G_2 population or, more likely, by a partial blockage in the G_2 phase due to cross-linking of completed DNA strands, thereby inhibiting mitosis [11].

The reduced incorporation of tritiated thymidine into DNA is indicative of an action of the drug, extending to both proliferative waves, on the G_0 phase of the cell cycle of the parenchyma. The lower specific activity of the liver DNA may also be due, in part, to an influence of the drug on the activities of thymidine and thymidylate kinases. The levels of the kinases follow the mitotic activity of the parenchyma [28] and are reduced in leukocytes from leukaemic patients after busulphan therapy [29]. Partial inhibition of the enzymes would account for the (small) increase in duration of the S phase of the parenchyma following busulphan treatment. Busulphan (or its derivatives) has been reported to decrease the incorporation of tritiated thymidine into the DNA of bone marrow cells [30] and murine leukaemic cells [31] *in vitro*, but to be without effect on the incorporation of the labelled precursor into DNA of mouse seminous epithelium [32], rat lens epithelium [11] and mouse thymocytes [33].

The slower restoration of liver weight for busulphan-treated rats during the first 30 hr following operation is unlikely to have been due to differences in glycogen content, resulting from reduced feeding, or to an effect on the increased fluid uptake following partial hepatectomy [13], since significant differences between control and experimental animals in DNA and RNA contents were not observed over this time period.

It is probable, therefore, that the drug arrests cells, directly or through action on the endoplasmic reticulum [34], in the resting, G_0 phase (or lowers the transition probability for transfer from the A-state to B-phase [35]), thus reducing the number of cells entering the proliferative state. An effect on the G_0 phase of the parenchymal cells should be apparent in reduced values of mitotic index for experimental animals, provided that the proportion of mitotic abnormalities is low and that they are evenly distributed within the tissue. In practice, a consistent reduction in the values of the mitotic index for experimental animals over the period 20–30 hr following operation could not be substantiated. A greater variation in these

values for drug-treated animals suggests, possibly, that mitotic abnormalities were not evenly distributed within tissue slices [11].

The duration of the G_1 phase following drug treatment was not obtainable from the labelled mitoses curve. An increase in the duration of this phase would depress further the rate of entry of cells into the S phase and cause the peak values for tritiated thymidine incorporation to occur at correspondingly later times. The time interval between measurements of thymidine incorporation was too long for a difference to be observed between control and experimental animals of < 3 hr in the peak rates of DNA synthesis for the first and second wave of proliferation. The symmetry of the thymidine incorporation curves make it unlikely, however, that the duration of the G_1 phase is appreciably affected by the drug.

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Oestrogen Receptor Activity and Endocrine Status in DMBA-Induced Rat Mammary Tumours

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Abstract—*Oestrogen receptor activity (unfilled sites) has been determined in DMBA-induced rat mammary tumours and at sacrifice, the plasma concentrations of oestradiol-17β and prolactin were determined by radioimmunoassay.*

In intact animals, receptor levels were found to fluctuate in inverse relationship with the plasma oestrogen concentration, but following ovariectomy, levels were significantly reduced. Administration of oestrogen to ovariectomized animals did not detectably change receptor levels but administration of perphenazine significantly increased receptor levels towards those seen in intact animals.

In animals bearing two or more tumours, similar receptor concentrations were detected in tumours from the same animal.

It is concluded that the concentration of oestrogen receptor sites in rat mammary tumours is influenced by the circulating levels of both oestrogen and prolactin.

INTRODUCTION

DMBA-INDUCED rat mammary tumours, like some human breast cancers, are hormone-sensitive [1]. They contain receptors for oestrogen [2–6], prolactin [7, 8] and progesterone [9]. The oestrogen receptors in these rat tumours have been shown to be similar to those in human breast cancers chemically [10] and in their relation to response to endocrine surgery [4]. Prior to evaluating further the relationship between receptor measurements and the endocrine responsiveness of experimental mammary tumours, we have investigated the influence of endocrine status on the level of receptor activity.

MATERIAL AND METHODS

Tumours

Female Sprague–Dawley rats were given DMBA (30 mg) by intragastric instillation. Tumours induced within 6 months were measured with calipers twice weekly. Size was expressed as the product of measurements in two dimensions. On reaching 1·5 × 1·5 cm tumours were either studied directly or subjected to endocrine manipulation prior to receptor assay. Tumours were excised after exsanguination of the rat under ether anaesthesia. Vaginal smears were taken daily from 45 intact animals and the stage in the oestrous cycle was classified as described by Yoshinaga *et al.* [11].

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Endocrine manipulation

Fourteen tumour-bearing rats were injected subcutaneously (s.c.) with perphenazine (5 mg/kg body weight) for 14 days.

A further 57 tumour-bearing rats were ovariectomized under ether anaesthesia and divided into three groups which were treated differently. In one group (OVX) of 11 rats, no further treatment was given until sacrifice 14 days later. In a second group (OVX+OE_2), 33 animals were left for 9–14 days and then injected s.c. with oestradiol 17-β (1–5 mg day in corn oil) for a further 6 days. In the third (OVX+Perphen) 13 animals were treated either immediately (9 rats) or after a period of 6–13 days of no treatment (4 rats) by subcutaneous injection of perphenazine (5 mg/kg body weight) for 6–14 days.

Multiple tumours in the same rat

In a total of 24 animals in various reproductive states (intact or endocrine manipulated),

oestrogen receptor activity was determined in two or more tumours from the same animal.

Determination of oestrogen receptor concentration

Tumour receptor concentrations were determined by a modification of a method described previously [12]. This modification was necessary to improve assay precision since by the original method, the low concentrations of receptor activity encountered in rat tumours lead frequently to non-linearity of the resulting Scatchard [13] graph.

Tumours were left in Tris buffer solution (0·25 M sucrose, 10 mM Tris and 1 mM ethylene diamine tetra-acetate pH 8·0) containing 0·5 mM dithiothreitol (14) for 1 hr at 4°C prior to homogenization at the rate of 300 mg/ml of Tris buffer and centrifugation of the homogenate at 105,000 *g* for 50 min (based on 15). The resulting cytosol was subjected to saturation analysis as previously described [12].

Determination of plasma hormone levels

Animals were exsanguinated at death through the abdominal aorta under ether anaesthesia, and the blood was used for the determination of plasma oestradiol-17β and plasma prolactin concentrations [16]. Since the quantity of blood collected was sometimes limited, assay sensitivity for plasma oestradiol-17β calculated for 4·0 ml plasma, was 0·13 ng/100 ml.

For plasma prolactin assay, ^{125}I-iodo-prolactin was prepared by the method of Redshaw and Lynch [17] a procedure which we found more reproducible than that previously employed. The sensitivity for 22 assays varied from 1 to 7·6 ng prolactin/ml with a mean value of 3·3 ng/ml. Inevitable deterioration of prolactin standard occurs on storage (NIAMDD instructions) and a correction for this has been made using the quality control data, a procedure which results in slightly lower values than those we reported previously.

Determination of cytosol protein concentrations

Protein concentration was determined by the method of Lowry, Rosebrough, Farr and Randall [18] using bovine serum albumin as standard.

Statistical analysis

Except where otherwise stated, statistical evaluation was carried out by the Wilcoxon rank test.

RESULTS

For most rat mammary tumours, the dissociation constant of binding (K_d) was found to be $0{\cdot}4$–$0{\cdot}5 \times 10^{-10}$ molar (see Table 1), in agreement with our earlier report [12].

The values found for tumour receptor concentration (P_0) by the method described in this paper are, however, $1{\cdot}67 \pm 0{\cdot}29$ (s.e., $N = 11$) times lower than those derived by the earlier method. By the modified method, a linear Scatchard plot was obtained for most of the tumours examined, and tumours with apparent receptor concentrations down to 0·05 fmoles/mg tissue clearly showed increasing displacement of (^{3}H) oestradiol-17β with increasing mass of non-radioactive oestradiol-17β added.

Effect of the oestrous cycle on tumour receptor concentration

In 45 cycling rats, tumour receptor concentration varied during the oestrous cycle (Fig. 1 and Table 1) reaching a minimum in proestrous which was significantly lower than the values in all other stages in the cycle ($P < 0{\cdot}01$), and

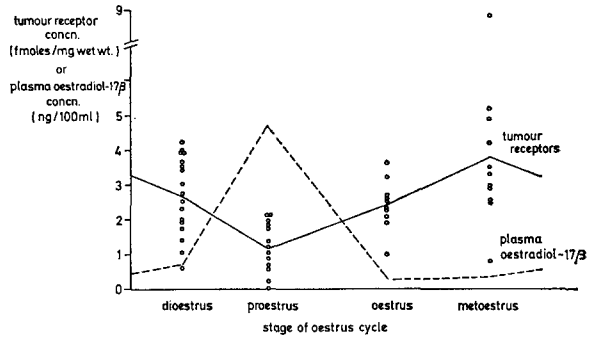

Fig. 1. The effect of stage in the oestrous cycle upon tumour oestrogen receptor concentration and plasma oestradiol-17β concentration. Each circle represents the oestrogen receptor concentration in an individual tumour. Lines join the mean receptor concentration (——) and mean plasma oestradiol-17β concentration (– – –). The standard errors for plasma oestradiol-17β concentration have been omitted for clarity but are to be found in Table 1.

a maximum in metoestrus. The changes in receptor concentration were in inverse relation to those in plasma oestradiol-17β (Fig. 1). The changes in plasma prolactin concentration followed, with a delay, those in oestradiol-17β but did not reach statistically significant levels.

Effect of ovariectomy and oestrogen administration

Following ovariectomy, tumour receptor concentrations (Fig. 2) and the plasma levels of both prolactin and oestradiol-17β (Table 1)

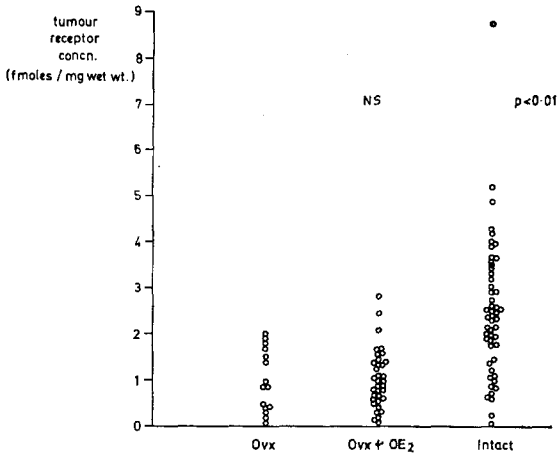

Fig. 2. The effects of ovariectomy and of ovariectomy followed by administration of oestradiol-17β on tumour receptor activity. The values for the tumours from the intact animals shown in Fig. 1 are reproduced for comparison. Each circle represents the value for an individual tumour P values refer to comparison with the concentrations in ovariectomised animals.

were significantly diminished relative to the values found in the tumours of intact animals ($P < 0{\cdot}01$).

Administration of oestrogen to ovariectomized rats caused an insignificant increase in detectable receptor activity (Fig. 2), and a restoration of prolactin levels to those seen in intact animals (Table 1).

Effect of perphenazine administration

Tumour concentrations of receptor activity in untreated rats were compared with the values in perphenazine-treated rats for both intact and ovariectomized states. The results are shown in Fig. 3 and Table 1.

In the intact animal, neither the mean tumour receptor concentration nor the plasma oestradiol-17β concentration after perphenazine-treatment was significantly different from the value found in untreated animals in dioestrus. Although perphenazine-treatment significantly increased the plasma prolactin concentration over the value found in untreated rats in dioestrus ($P < 0{\cdot}01$), the increased concentration was not significantly greater than the highest concentration observed in untreated animals at proestrus.

In ovariectomized animals, perphenazine-treatment caused a significant elevation in plasma prolactin concentration and in tumour receptor concentration ($P < 0{\cdot}01$), whilst dissociation constant of oestrogen binding by the

Table 1. Tumour receptor concentrations and plasma hormone levels in tumour-bearing rats in various endocrine states

Reproductive state	No. of rats	No. of tumours	Tumour receptor activity: K_d ($\times 10^{-10}$M)	P_0 (fmoles/mg) (tissue)	P_0, protein (fmoles/mg) (protein)	Plasma: Oestradiol-17β (ng/100ml)	Prolactin (ng/ml)
Sensitivity*			—	0·05	1	0·13	3
Intact proestrus	10	13	0·64 ± 0·10	1·21 ± 0·20	34 ± 7	4·70 ± 0·69	54 ± 16
oestrus	9	10	0·53 ± 0·04	2·42† ± 0·23	58‡ ± 8	0·41† ± 0·15	51 ± 7
metoestrus	11	11	0·40§ ± 0·03	3·77† ± 0·60	94‡ ± 25	0·32† ± 0·07	35 ± 6
dioestrus	13	17	0·49 ± 0·05	2·70† ± 0·27	70 ± 11	0·70† ± 0·17	26 ± 6
Intact + perphenazine (dioestrus)	14	19	0·39 ± 0·03	3·11 ± 0·30	72 ± 11	0·43 ± 0·08	77† ± 16
Ovariectomised OVX	11	15	0·72 ± 0·11	0·94 ± 0·17	23 ± 5	0·13 ± 0·02	12 ± 2
OVX + OE_2	33	36	0·62 ± 0·05	1·00 ± 0·11	22 ± 3	0·26 ± 0·05	40† ± 6
OVX + perphenazine	13	16	0·42† ± 0·005	1·86 ± 0·19	44 ± 6	0·16 ± 0·03	59† ± 11

Results are the mean values ± 1 standard error.
*Sensitivity defined as described under "Methods and Materials" and first section of results.
†$P < 0{\cdot}01$, ‡$P < 0{\cdot}02$, §$P < 0{\cdot}05$ when compared with appropriate reference value:
during oestrous cycle, values compared with that in proestrus; "Intact + perphenazine" values compared with "intact-dioestrus" value; "OVX + OE_2" and "OVX + perphenazine" values compared with "OVX" value.

tumour was significantly reduced ($P < 0{\cdot}01$) following perphenazine-treatment. No differences were noted between the values found for rats treated immediately after ovariectomy and those in which the start of treatment was delayed: accordingly the data for these two, slightly different modes of treatment have not been separated.

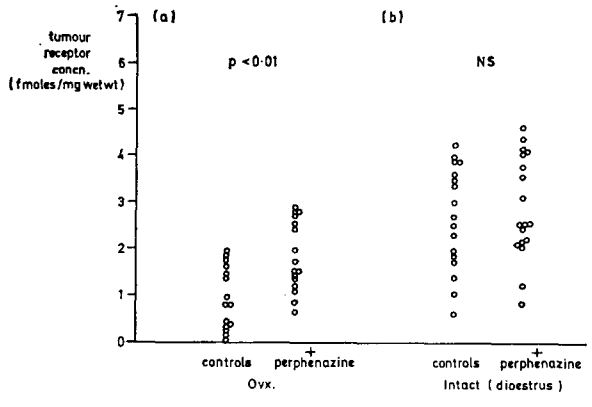

Fig. 3. The effect of perphenazine-administration in vivo upon tumour oestrogen receptor concentration in (a) ovariectomised and (b) intact rats. Each circle represents the value from an individual tumour. P values refer to the effect of perphenazine within each endocrine state.

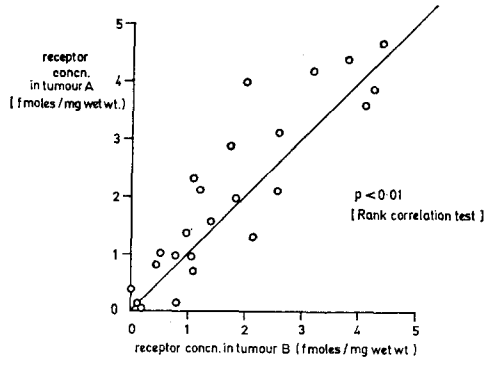

Fig. 4. Tumour receptor concentrations in rats bearing 2 tumours: receptor concentration for one tumour, A is plotted against that for the second tumour, B from the same rat. The rats were sacrificed in various reproductive states. Each circle represents the value from a single rat.

Receptor concentrations in multiple tumours from the same rat

When two or more tumours from the same animal were examined, the receptor concentration found for one tumour ("A") was more or less identical to the value for a second tumour ("B") from the same animal (Fig. 4). For animals with more than two tumours, only two values, selected by random selection (tables of random numbers), have been plotted.

In most cases, tumours from the same animal contained very similar concentrations of oestrogen receptors. By the Rank Correlation test, there was a highly significant ($P < 0{\cdot}01$) correlation between the receptor levels in tumours from the same animal.

DISCUSSION

Oestrogen receptor activity has been determined in mammary tumours of the rat in various endocrine states. It was found necessary to modify the method previously employed [12] in three respects (based on 14 and 15) to permit accurate determination of the relatively low concentration of receptor binding sites present in these tissues. By this modified method, oestrogen receptor activity was detected in 98·5% of the 136 DMBA-induced mammary tumours examined. The receptor activity in these tumours was characterized by a dissociation constant of binding ranging from $0{\cdot}16\text{–}1{\cdot}80 \times 10^{-10}$ molar, and in most tumours dissociation constant was in the range 0·4–$0{\cdot}5 \times 10^{-10}$ molar.

The range of oestrogen receptor concentrations encountered in rat mammary tumours is approximately ten times lower than that found in human breast cancers [12]. A similar difference is seen between the concentrations of plasma oestradiol-17β in the two species [16, 19].

Although most methods for determining oestrogen receptor concentrations detect only empty receptor sites, in general workers do not report the stage in the cycle at which tumours have been harvested for receptor assay or estimate plasma oestrogens at the time the tumour is harvested. We have shown that receptor concentrations fluctuate in inverse relationship with the prevailing oestradiol-17β concentration, and that the tumour receptor concentration is a function of the stage in the oestrous cycle at which the tumour was excised.

This finding is in contrast to the recent work of Jordan and Jasper [20] who conducted receptor assays at 30°C, a temperature at which exchange between (^{3}H) oestradiol-17β and endogenous oestrogen at filled receptor sites might occur. However, our results agree with those of Lee and Jacobson [21] and of Trams, Engel, Lehmann and Maass [22] in the rat and human uterus respectively, and of Maass, Engel, Nowakowski, Stolzenbach and Trams [23] in human breast cancer.

It is known that the effects of oestrogen upon the oestrogen receptor concentration within a tissue are complex: cytoplasmic receptor levels can be altered by increases in circulating oestrogen in two ways. In addition to (i)

filling empty receptors, which are then translocated to the cell nucleus, oestrogen may also (ii) stimulate the synthesis of new receptors [24, 25]. Our observations that tumour receptor levels firstly vary during the oestrous cycle and secondly, fall after ovariectomy, are, respectively, in keeping with these two effects.

In the rat, changes in circulating oestrogen level can also lead to alterations in the concentration of plasma prolactin (see e.g. [26].) This hormone has also been reported to influence oestrogen receptor levels in the rat mammary tumour [27–29]. In view of this, it is difficult to ascribe changes in receptor level to the effect of a single hormone. Nevertheless, in intact cycling animals, the tumour levels of detectable receptor activity found in this study were inversely related to changes in circulating oestradiol-17β concentration and apparently unrelated to changes in plasma prolactin concentration.

In the ovariectomised rats treated with perphenazine, tumour receptor levels were clearly elevated in association with a significant increase in prolactin secretion, but in the absence of detectable oestrogen. This effect of prolactin on oestrogen receptor levels is in agreement with the findings of Leung and Sasaki [27, 28] and of Vignon and Rochefort [29]. Although perphenazine-treatment in ovariectomised rats also increased the affinity of the receptors for oestrogen (see Table 1), this apparent difference probably reflects the less accurate determination of Kd at low receptor levels rather than a specific effect of prolactin.

In ovariectomised rats treated with oestrogen, tumour receptor levels were not detectably changed relative to untreated controls, possibly due to the filling of empty sites by the injected oestrogen. The failure to detect any effect of perphenazine-treatment upon tumour receptor levels in intact animals may either be due to the fact that the plasma prolactin concentrations found after treatment were not significantly greater than those found in untreated animals at proestrus and oestrus, or may indicate that the latter concentrations are already sufficient to maximally stimulate tumour receptor formation.

In animals bearing two or more tumours, despite the existence of a different clone of cells in each tumour, receptor levels were strongly correlated between tumours from the same animal, indicating that the endocrine milieu may be the major determinant of tumour receptor level.

These results support the view that endocrine status markedly influences tumour receptor level. In particular, we have indicated that oestrogen receptor levels may be increased by prolactin or reduced by the filling of empty receptor sites with oestrogen. If a similar situation obtains in women with breast cancer, then endocrine status at biopsy for receptor assay would influence the receptor levels detected and hence the predictive value of this parameter. The adoption of an exchange assay for measuring total cellular receptors should help by reducing the effect of circulating oestrogens on detectable receptor level for (i) clinical, predictive purposes and (ii) separating the effects of oestrogen and prolactin on receptor levels in the rat mammary tumour model.

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Effects of BCG and *Corynebacterium Parvum* on the Haemopoietic Precursor Cells in Continuously Irradiated Mice: Possible Mechanisms of Action in Immunotherapy*

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Abstract—*Single or repeated administration of BCG or* Corynebacterium Parvum (C. Parvum) *to continuously irradiated mice resulted in increased numbers of haemopoietic precursor cells in their bone marrow and spleen. These cells were detected by their ability to form nodules in the spleen of irradiated transplanted recipients or monocyte/granulocyte colonies in agar diffusion chambers. This increase in haemopoietic progenitor cells did not, however, confer any advantage, in terms of survival when the mice were challenged with further doses of irradiation. The relevance of these findings to the available clinical data is discussed.*

INTRODUCTION

AGENTS, such as BCG and *C. Parvum*, have been used as non-specific stimulators of the immune system in cancer immunotherapy in man and in experimental animals [1]. These agents have also been shown to stimulate macrophage production [2–5] and these cells play an important part in the action of the host against the tumour [6]. Macrophages are derived from monocytes which are produced in the bone marrow and they have also been shown to be closely related to granulocyte production [7]. For this reason we decided to investigate the effects of BCG and *C. Parvum* on the haemopoietic stem cells in mice.

Mathé [8] found that immunotherapy in mice was successful only if the burden of tumour cells was very small and suggested that leukaemia would be an ideal situation for the investigation of immunotherapy in man [9]. Prior to remission, patients receive a considerable amount of cytoxic chemotherapy which results in marked marrow hypoplasia. Although the patients regain adequate peripheral blood counts during immunotherapy maintained remission, their marrow tends to remain hypoplastic [10].

For this reason we investigated the effects of BCG and *C. Parvum* on the haemopoietic colony forming cells in mice whose marrow had been depleted by continuous exposure to ^{137}Cs irradiation at approximately 35 rad/day for 1–2 weeks [11]. Accordingly, groups of mice were maintained under continuous irradiation and treated with BCG or *C. Parvum* as single or repeated doses. The effects of BCG or *C. Parvum* on the marrow were measured using the spleen colony assay [12] and the agar diffusion chamber technique [13]. In other experiments, the ability of mice treated with BCG or *C. Parvum* to survive further acute or chronic doses of irradiation was compared with that of untreated control groups.

The results show that although BCG and *C. Parvum* exert stimulatory effects on haemopoietic precursor cells this does not increase their survival after further treatment.

MATERIAL AND METHODS

Experimental animals

C57 Bl mice were used in all experiments.

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At the beginning of the experiments they were between 2 and 3 months old.

BCG and C. Parvum

BCG (Glaxo Ltd.) was injected intradermally (approx. 10^7 live organisms) while *C. Parvum* (Wellcome Research Laboratories) was injected intraperitoneally (0·35 mg dry weight/mouse).

Irradiation facilities

Continuous irradiation (CI) was carried out in a ^{137}Cs unit. The required dose rates for the experiments (38 or 100 rad/day) were achieved by varying the distance of the animals from the central source. Acute doses of radiation were delivered from a ^{60}Co source at a dose rate of approximately 60 rad/min.

Assay systems

Pluripotential haemopoietic stem cells were measured using the spleen colony assay of Till and McCulloch [12] and the committed granulocytic stem cells were detected by their ability to form colonies (ADC-C) in agar diffusion chambers [13].

RESULTS

The effects of continuous irradiation (CI) alone on the spleen colony forming cells (CFU-S) and the granulopoietic cells forming colonies in agar diffusion chambers (ADC-C) are shown in Fig. 1. Both cell populations measured by these assays were depleted by CI; the CFU-S reached a new steady state of approximately 5% of normal values within 1 week after the onset of irradiation and then maintained this level while the ADC-C showed an initial rapid decline to 30% of control values followed by a slower fall to 6% over the 5 week period.

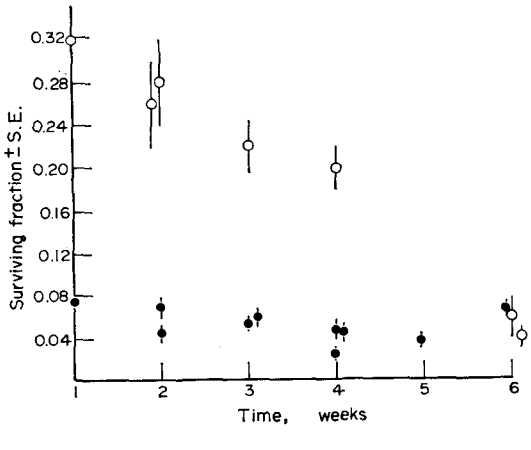

Fig. 1. The effect of CI on the CFU-S (●) and the ADC-C (○) in C57B1 mouse marrow.

Effects of BCG and C. Parvum *on CFU-S*

Figures 2(a) and 2(b) show the effects of BCG or *C. Parvum*, either as single or as repeated weekly doses, on the CFU-S in the bone marrow and spleen of CI mice. The results are expressed in terms of colony forming cells per femur, or per spleen, relative to the number of colony forming cells in mice treated with CI alone. Single doses of BCG or *C. Parvum* resulted in a two-fold increase in bone marrow CFU-S one week after administration followed by a decline to less than the control

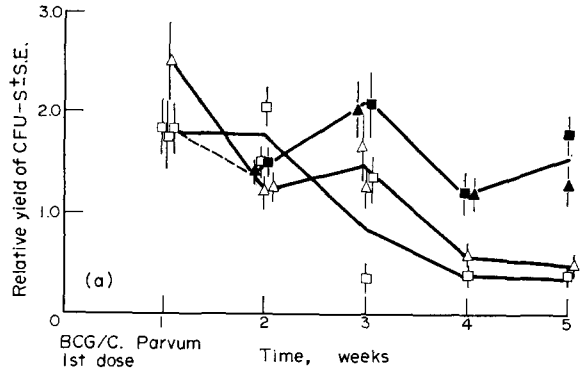

Fig. 2(a). The effects of BCG (■) and C. Parvum *(▲) on the CFU-S in the marrow of CI mice. Open symbols refer to single doses, closed symbols refer to weekly doses.*

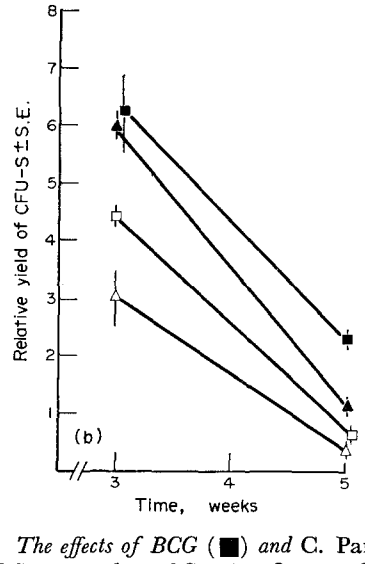

Fig. 2(b). The effects of BCG (■) and C. Parvum *(▲) on the CFU-S in the spleen of CI mice. Open symbols refer to single doses: closed symbols refer to weekly doses.*

(CI only) value by the 5th week. Weekly doses of BCG or *C. Parvum* maintained the levels of CFU-S above those observed with single doses but there was no increase in their number as the number of doses was increased.

The numbers of CFU-S in the spleen were measured only at 3 and 5 weeks after single or repeated doses of BCG or *C. Parvum*. Much greater increases were seen in the spleen than in the bone marrow, relative to the effects of CI alone. At 3 weeks the single doses resulted in a 3–4 fold increase and repeated doses gave a 6-fold increase but by the 5th week the measured values approximated the control levels.

Effects of BCG and C. Parvum *on ADC-C*

Figures 3(a) and 3(b) show the responses of the committed granulopoietic progenitor cells

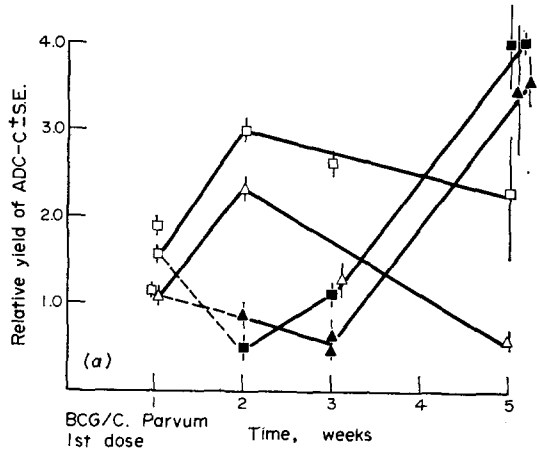

Fig. 3(a). The effects of BCG (■) and C. Parvum (▲) on the ADC-C in the marrow of CI mice. Open symbols refer to single doses; closed symbols refer to weekly doses.

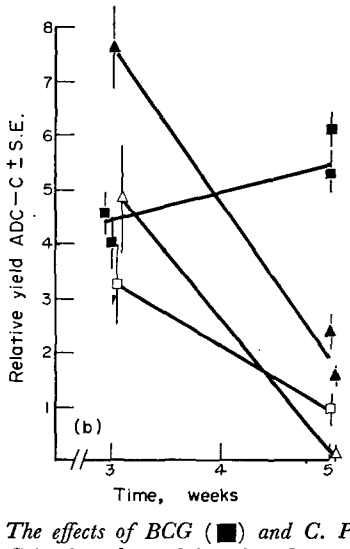

Fig. 3(b). The effects of BCG (■) and C. Parvum (▲) on the ADC-C in the spleen of CI mice. Open symbols refer to single doses; closed symbols refer to weekly doses.

in the bone marrow and spleen of CI mice to treatment with BCG or *C. Parvum*. The time pattern of the bone marrow response to these agents, measured using the ADC technique, differed from that seen with the CFU-S assay in that the greatest numbers following single doses were seen at 2 weeks, not one week. after administration. At 2 and 3 weeks the effect of repeated weekly doses was less than that seen 2–3 weeks after a single dose, but by the 5th week the continuation of weekly doses had resulted in a four-fold increase which was greater than the residual effect of a single dose given 5 weeks earlier.

The response of ADC-C in the spleen 3 and 5 weeks after a single dose of BCG or *C. Parvum*, or repeated *C. Parvum*, was similar to that seen for spleen derived CFU-S. However, a difference was seen after repeated doses of BCG when the splenic ADC-C level was higher at 5 weeks than at 3 weeks.

To determine whether the increases in progenitor cell levels resulting from treatment with BCG or *C. Parvum* prolonged animal survival after further challenge, treated or control groups of mice were subjected to either chronic (100 rad/day) or acute (60 rad/min) doses of irradiation. For the chronic irradiation survival experiment groups of animals were given BCG or *C. Parvum* at weekly intervals during exposure while the control groups were subjected to CI alone. For the acute irradiation survival experiment, mice were exposed to CI for 2 weeks during which time they were given BCG, *C. Parvum* or no treatment. A further dose of BCG or *C. Parvum* was given to the treated animals 1 day after exposure to 900 rad ^{60}Co γ-irradiation.

The graphs of animal survival following CI or acute irradiation show that the apparent improvement in marrow status resulting from treatment with BCG or *C. Parvum* did not markedly improve their ability to withstand further insult. In the case of chronic irradiation (Fig. 4), treatment with BCG or *C. Parvum* slightly delayed the onset of death, thus increasing the mean lifespan of the animals, but the maximum survival in each group was the same (45–46 days). Pretreatment with CI alone improved animal survival following acute irradiation when compared with the survival of normal animals given 900 rad (Fig. 5). However, the animals which had received BCG or *C. Parvum* in addition to CI died sooner than the untreated CI controls. In fact, those animals treated with CI + BCG survived less well than the normal animals given 900 rad.

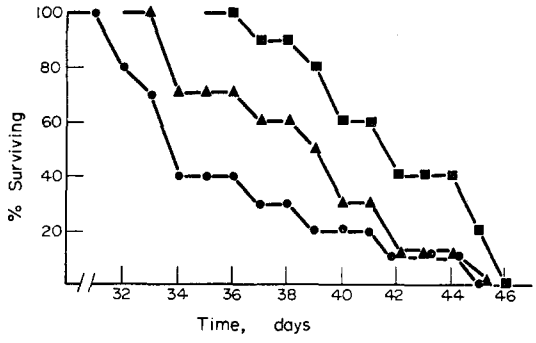

Fig. 4. The effects of BCG (■) and C. Parvum *(▲) on the survival of CI mice compared with the survival of mice receiving CI alone (●).*

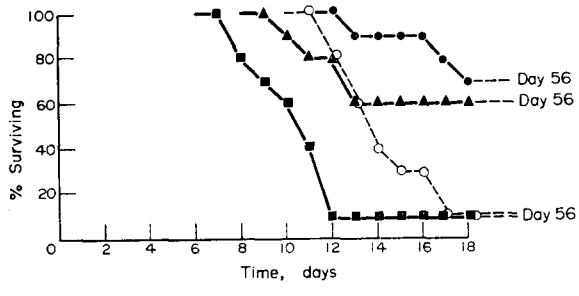

Fig. 5. The effects of BCG (■) and C. Parvum *(▲) on the survival of CI mice given acute irradiation compared with the survival of mice pretreated with CI alone (●) or given no pretreatment (○).*

DISCUSSION

The present study was carried out to determine whether treatment with BCG or *C. Parvum* could increase the number of haemopoietic progenitor cells in mice subjected to continuous irradiation and whether any increase in these cells improved survival following a second insult. The rationale for these experiments is based on the suggestion that the prolonged survival of patients maintained on immunotherapy during acute myeloid leukaemia in remission results, at least in part, from a non-specific immuno-stimulation of the marrow precursor cell pool; such stimulation of haemopoietic precursor cells may allow patients who had relapsed better to withstand the myelotoxic effects of reinduction chemotherapy [14].

Most published reports have concentrated on the effects of BCG and *C. Parvum* on macrophage production in the bone marrow using *in vitro* systems for the growth of macrophage colonies. These studies have also looked at relatively short time periods but, since patients are maintained on immunotherapy for months or even years [15] we attempted a longer term investigation of the effects of these agents. Furthermore, previous work has been done using tumour bearing animals, but the continuously irradiated mouse may more closely parallel the situation in immunotherapy patients since in both cases adequate peripheral blood counts are maintained in spite of bone marrow depletion [10].

The work of Wolmark and Fisher [2], Fisher *et al.* [3], Dimitrov *et al.* [4] and Baum and Breese [5] supports the idea that BCG and *C. Parvum* stimulate precursor cells in the bone marrow to increase the production of macrophages. In these studies the longest time interval investigated was 10 days after a single injection of *C. Parvum* into normal mice [5], or 18 days after injection of BCG into normal or tumour bearing mice [3]. Dimitrov *et al.* [4] also investigated the effect of *C. Parvum* on the levels of colony stimulating activity in serum and found an inhibitory effect. This finding is in agreement with our results which failed to detect any humoral stimulation when animals treated with BCG or *C. Parvum* were used as hosts for agar diffusion chambers containing normal mouse bone marrow (Gordon—unpublished data).

The present studies have shown that BCG and *C. Parvum* can maintain haemopoietic precursor cell levels above control (CI only) values, in spite of the cell killing effects of continuous irradiation, for up to 5 weeks. However, this increase does not markedly improve animal survival following continuous irradiation and reduces survival following acute irradiation.

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Immunogenicity Induced *In Vivo* by DIC in Relatively Non-Immunogenic Leukemias*

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Abstract—*Following a cycle of treatment with the compound* 5-(3, 3-*dimethyl*-1-*triazeno*)*imidazole*-4-*carboxamide* (*DIC*), *two murine tumors, the chemically induced EL4 leukemia and the Gross-virus induced GL leukemia evidenced new immunogenic properties in syngeneic hosts. The increased survival-time and the increased effectiveness of a single BCNU treatment of animals challenged with the drug-treated cells, relative to that observed in animals challenged with parental, drug-untreated tumors or in immunosuppressed animals challenged with DIC-treated leukemias provide indirect evidence for increased immunogenicity of the tumor cells.*

In experiments involving immunization of syngeneic animals with X-ray-inactivated DIC-altered cells, clear evidence for a specific primary and a specific secondary immune-response was obtained. As observed previously in studies with other experimental tumors, the immunogenic properties were retained by the drug-treated cells over a long series of transplantations in normal or immunosuppressed syngeneic animals.

INTRODUCTION

IT HAS been reported [1–7] that treatment of tumor-bearing animals with antineoplastic compounds can lead to modification of tumor cell immunogenicity as evidenced by the prolonged or indefinite survival of syngeneic hosts challenged with the drug-treated neoplasms. An immune response to the drug-modified cells has been observed both in *in vivo* [8, 9] and in *in vitro* experiments [10, 11]. As the drug-induced alteration persisted indefinitely after the withdrawal of the chemotherapeutic treatment, the antigenic sublines are considered of interest for cancer cell biology and for experimental immunotherapy.

Most of the studies pertaining to drug induced alteration of tumor cell immunogenicity have been performed with lymphomas that originated in DBA/2 mice (H-2^d), and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DIC) has been among the most active compounds.

In the current study, alteration of the immunologic properties of two ascitic murine leukemias, the chemically-induced EL4 lymphoma in C57Bl/6 mice (H-2^b) and a Gross-virus induced leukemia in C3H mice (H-2^k), was obtained following *in vivo* treatment with DIC. These leukemic lines were chosen since they differ in the major H-2 histocompatibility region from the previously studied DBA/2 lymphomas. Also, in preliminary experiments, they failed to evoke any appreciable immuno-response in syngeneic hosts.

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MATERIAL AND METHODS

Animals and tumors

Inbred C3H/HeCr and hybrid (DBA/2 ♀ × C57Bl/6 ♂) F_1 male mice, hereafter called BDF_1, 6 to 10 weeks old, obtained from the Mammalian Genetics and Animal Production Section of the National Cancer Institute, NIH, were used. The chemically induced EL4 lymphoma and the Gross virus induced leukemia (GL), obtained as previously indicated [11], were maintained by intraperitoneal (i.p.) passage of 10^6 ascitic cells in BDF_1 and C3H mice respectively.

For the development of DIC-sublines the procedure previously described [3] was fol-

lowed with minor modifications. Briefly, 10^7 EL4 cells and 10^6 GL cells were injected i.p. (day 0) into compatible animals. BDF_1 mice inoculated with EL4 cells were treated with 100 mg/kg i.p. of DIC for 10 consecutive days, starting one day after leukemic inoculation. C3H mice inoculated with GL were treated for 5 consecutive days, also starting one day after leukemic inoculation; drug treatment was then discontinued for two days and resumed for three additional days. EL4 lymphoma was treated for 12 transplant generations and GL leukemia for 8 transplant generations. Once established, the DIC-sublines were maintained in mice immunosuppressed with cyclophosphamide (200 mg/kg, administered i.p. 24 hr before tumor challenge). Cell viability was checked by dye-exclusion and inocula with at least 90% viability were employed.

Chemicals

DIC (NSC-45388) was dissolved in chilled saline immediately before use after addition of equal parts (w/w) of citric acid; BCNU (NSC-409962) was dissolved in a few drops of ethyl alcohol and the volume adjusted with saline; cyclophosphamide (NSC-26271) was dissolved in saline. The drugs were obtained through the courtesy of Dr. H. B. Wood, Jr. (Drug Research and Development Program, National Cancer Institute, Bethesda, Md., U.S.A.).

Immunization

Single i.p. injections of 4×10^7 X-irradiated cells (5000 R—200 kV, 12 mA, 0·5 mm Cu–Al filter, 100 R/min) were given 21 days before challenge with viable cells.

RESULTS

The occurrence of drug-mediated increase in immunogenicity was tested by injection of tumor cells from DIC treated tumors at each successive transplant generation into normal or immunosuppressed hosts. The survival was then followed for ninety days. For both the drug treated EL4 leukemia (EL4/DIC) and the GL leukemia (GL/DIC), when injected into normal recipients there was a progressive increase in survival time following successive generations of treatment as compared with previously untreated parental leukemic cells. For the EL4 leukemia (EL4/DIC) the maximum increase in median survival time (MST) was obtained

Table 1. EL4/DIC titration in normal and immunodepressed mice

Tumour challenge (i.p.)	Normal animals						X-irradiated animals					
	EL4			EL4/DIC			EL4			EL4/DIC		
	MST	(Range)	D/T	MST	(Range)	D/T	MST	(Range)	D/T	MST	(Range)	D/T
10^2	43	(33–50)	7/8	—	(–)	0/8	21	(17–18)	6/6	31	(23–43)	4/6
10^3	41	(28–43)	8/8	—	(–)	0/8	20	(17–21)	5/5	31	(5–31)	3/5
10^4	31	(29–33)	8/8	—	(58–69)	3/7	15	(7–21)	6/6	35	(16–38)	6/6
10^5	26	(23–31)	7/7	55	(39–55)	6/8	12	(6–18)	6/6	28	(5–35)	5/6
10^6	20	(18–24)	8/8	40	(39–56)	8/8	10	(9–15)	6/6	24	(20–28)	6/6
10^7	15	(14–15)	8/8	45	(42–57)	8/8	11	(9–11)	6/6	22	(14–25)	6/6

Table 2. GL/DIC titration in normal and immunosuppressed C3H mice

Challenge i.p.	Normal animals						X-irradiated animals					
	GL			GL/DIC			GL			GL/DIC		
	MST	(Range)	D/T	MST	(Range)	D/T	MST	(Range)	D/T	MST	(Range)	D/T
10^2	20	(17–48)	6/8	59	(32–59)	5/8	19	(13–30)	6/6	22	(12–24)	6/6
10^3	17	(15–20)	8/8	58	(33–64)	7/7	17	(16–20)	6/6	22	(11–36)	5/5
10^4	16	(14–17)	7/7	51	(36–58)	7/8	17	(14–17)	7/7	22	(21–34)	6/6
10^5	14	(12–16)	7/7	42	(33–52)	8/8	12	(11–15)	6/6	21	(21–22)	6/6
10^6	12	(11–15)	8/8	41	(30–49)	7/7	11	(11–14)	6/6	13	(9–15)	6/6
10^7	10	(7–10)	8/8	53	(36–54)	6/8	11	(11–11)	5/5	14	(13–15)	6/6

on transplant of the tumor into normal recipients following DIC treatment for 12 transplant generations (Table 1). For the GL leukemia (GL/DIC) the maximum effect was observed after 8 transplant generations (Table 2). Challenge of normal mice with tumor cells from animals treated with DIC for more than 12 or 8 transplant generations respectively did not produce any further increase in the survival time of the animals.

The EL4 leukemia was naturally insensitive to the DIC treatment, so that the MST of EL4 leukemic mice treated with DIC did not change significantly in the course of treatment over a series of transplant generations. The GL leukemia, which was originally sensitive to the chemotherapeutic activity of DIC, became progressively more resistant as reflected in a reduction of the MST treated animals.

For both the EL4/DIC (Table 1) and GL/DIC (Table 2) sublines a decrease in MST was observed in immunosuppressed animals. This finding, in agreement with previous data obtained with other immunogenic sublines, indicates that the increased survival of animals challenged with the drug-treated sublines is dependent upon the presence of an intact immune apparatus in the host.

It is of interest, but not clear as to why immunodepressed mice bearing the EL4 leukemia succumbed in a shorter time than the corresponding normal animals, despite the failure of the tumor to exert any appreciable antigenic effect in syngeneic animals.

Further evidence for the occurrence of an immune reaction of the host to a DIC-subline was obtained in the experiment shown in Table 3. BCNU (20 mg/kg) was administered as a single s.c. treatment on day 6 to normal or immunosuppressed animals challenged with the parental or EL4/DIC leukemia. The synergistic increase in MST of animals bearing the EL4/DIC leukemia, resulting from the chemotherapeutic treatment plus host immunoresponse [3], was reversed following reduction of animal immunoreactivity by X-irradiation (400 R).

Direct evidence for drug-induced immunogenicity of EL4 leukemia is provided by the specific immunization of syngeneic mice to the DIC treated subline. X-ray-inactivated EL4/DIC cells inoculated into compatible BDF_1 mice elicited specific sensitization as evidenced by animal survival to the challenge of EL4/DIC viable cells (Table 4). The low immunogenicity of the parental EL4 leukemia and the absence of immunogenicity of GL leukemia indicate that the treatment with DIC has induced new immunologic properties not detectable in the original leukemias that had not been subjected to treatment with the drug.

It has been demonstrated that an appropriate dose of X-rays may inhibit primary but not secondary immune response [12]. An experiment was conducted in which animals previously immunized with inactivated EL4 or EL4/DIC tumor cells were X-irradiated 24 hr before challenge with viable EL4 or EL4/DIC cells (Table 5). Further evidence for specific drug-induced immunogenicity is provided by the observation of specific sensitization by EL4/DIC in animals that were X-irradiated prior to secondary challenge with EL4/DIC. Increased survival of the mice was observed only in that group following challenge with viable leukemic cells.

DISCUSSION

In previous studies L1210 and L5178Y lymphomas, both originally chemically-induced in DBA/2 mice, are the tumors that have been employed primarily to obtain antigenic alteration and to investigate the new immunologic properties.

As *in vivo* immunologic alteration of tumor cells might be of some interest to exploit as a new tool for the specific immunotherapy of experimental tumors, it appeared to be important to determine the reproducibility of the previous findings with a wider range of tumors.

The difference in genetic origin of the EL4 ($H\text{-}2^b$) and Gross-virus induced ($H\text{-}2^k$) leukemias as compared with L1210 and L5178Y ($H\text{-}2^d$), the low immunogenicity of EL4 leukemia and the virus origin of the GL leukemia have been considered as properties of interest.

In the present studies increased immunogenicity of the two leukemias has been obtained following *in vivo* treatment with DIC. Indirect evidence, such as the increased survival time of normal animals bearing the drug-treated tumors and its partial reversal by immunodepression, the synergism between host immunoresponse and chemotherapeutic treatment, and direct evidence involving specific immunization assays, have substantiated the new immunologic properties in EL4/DIC and GL/DIC tumors.

In comparison with the previous DIC-treated DBA/2 lymphomas some minor differences have been observed. The increased immunogenicity was reached with a longer delay than that obtained with L1210 and L5178Y ($H\text{-}2^d$) lymphomas. Furthermore, a

Table 3. BCNU treatment of animals challenged with EL4/DIC subline

				BCNU (day +6)			400 R (day minus 1) and BCNU (day +6)		
	MST	(Range)	D/T	MST	(Range)	D/T	MST	(Range)	D/T
EL4	17	(12–20)	6/6	21	(16–24)	6/6	18	(15–22)	6/6
EL4/DIC	42	(36–54)	6/6	—	(30)	1/6	26	(14–31)	6/6

Table 4. EL4/DIC rejection by BDF_1 immune animals

Challenge i.p.	Animals immune to EL4						Animals immune to EL4/DIC					
	EL4			EL4/DIC			EL4			EL4/DIC		
	MST	(Range)	D/T	MST	(Range)	D/T	MST	(Range)	D/T	MST	(Range)	D/T
10^3	32	(18–56)	6/6	—	(60–62)	2/6	31	(25–42)	5/6	—	(57)	1/6
10^4	36	(31–44)	5/6	—	(43–67)	3/6	27	(25–37)	6/6	—	(35–67)	2/6
10^5	29	(26–33)	6/6	—	(35–65)	2/6	27	(22–41)	6/6	—	(31)	1/6
10^6	18	(16–23)	6/6	58	(38–63)	5/5	18	(15–21)	5/5	—	(27–36)	2/6
10^7	15	(15–17)	6/6	43	(31–54)	5/6	16	(15–23)	6/6	—	(33–39)	3/6

Table 5. EL4/DIC rejection by X-irradiated BDF_1 immune animals

Challenge i.p.	Animals immune to EL4						Animals immune to EL4/DIC					
	EL4			EL4/DIC			EL4			EL4/DIC		
	MST	(Range)	D/T	MST	(Range)	D/T	MST	(Range)	D/T	MST	(Range)	D/T
10^3	—	(28–41)	3/6	—	(23)	1/6	20	(17–24)	5/6	—	(—)	0/6
10^4	24	(21–29)	6/6	—	(18)	1/6	21	(20–29)	6/6	—	(35)	1/6
10^5	17	(16–26)	6/6	32	(22–41)	4/6	17	(14–21)	6/6	—	(—)	0/6
10^6	19	(11–25)	6/6	24	(16–28)	5/6	12	(10–15)	6/6	—	(42)	1/6
10^7	12	(11–15)	6/6	16	(12–22)	6/6	11	(10–15)	6/6	—	(—)	0/6

Animals have been irradiated (400 R) 24 hr. before tumor challenge.

less extensive host-immune reaction was elicited by the EL4/DIC and GL/DIC tumors. Animals bearing these DIC-altered leukemias showed increased survival time but except at reduced inoculum levels failed to reject the neoplasms completely.

Nevertheless, *in vivo* induction of tumor immunogenicity did occur with these additional leukemic lines. Also, the possibility exists that with other schedules of treatment with DIC or with other antineoplastic compounds immunologic alterations may be achieved which will be sufficiently extensive to induce definitive tumor rejection of these leukemias by a syngeneic host.

It has been suggested [13] that the inhibition of naturally occurring immunoselection by antineoplastic drugs might permit the growth of immunogenic clones. In regard to this it may be noted that the immunogenic property of the drug-altered leukemic cells has been maintained over a long period of time (100 transplant generations) both in normal as well as immunosuppressed mice. If the assumption of selection of immunogenic clones were true it might have been expected that over a few transplant generations in normal animals, a loss of the immunogenicity would occur.

The mechanism by which DIC or other drugs produces the immunologic alterations on tumor cells is still unknown. Several hypotheses have been advanced and discussed in previous papers [3–7]. In any event, the possibility of the drug acting as a hapten is irreconcilable with the stability and heritability of the cellular modification.

Experimental models for study of immune response by syngeneic hosts against two tumor-cell populations, the original and a subline of

differing immunogenicity obtained by pharmacologic manipulation, provide interesting possibilities for investigation. The utilization of drug-induced immunogenicity for experimental immunotherapy remains to be exploited.

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Organ Distribution of Exogenous Murine Mammary Tumour Virus as Determined by Bioassay*

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Abstract—*Cell-free mammary tumour virus activity was exhibited by extracts from the following organs of exogenously infected BALB/cfC3H mice: salivary gland, kidney, testis and epididymis. This was tested for by bioassay in female BALB/c mice, which were then forced bred for mammary tumour production. Mammary tumour inducing activity was demonstrated for intact cells of thymus, spleen, bone marrow and liver. Radiation* (3000 *rad*) *largely destroyed the cell-bound viral activity. No cell-free or cell-bound activity was found in lung, brain or pancreas. Epigenetic factors seem to have a great influence on the expression of exogenous mammary tumour virus.*

INTRODUCTION

THE MILKBORNE murine mammary tumour virus (MTV-S [1]) can be recovered not only from milk and mammary glands but also from blood and hemopoietic tissues and male secondary sex organs (for a review, see ref. [2]). The virus is known to transform only mammary cells. The mammary tumour virus, therefore, provides a good model for a study of the role of epigenetic factors in the expression of various genes of the murine mammary tumour virus. For that reason, we have begun an extensive research program on the organ distribution of MTV-S, employing electron microscopical, biochemical and immunological methods. This presentation is a report on the results of bioassays done on several organs of BALB/c mice which were exogenously infected with MTV-S.

MATERIAL AND METHODS

A chronically infected BALB/c mouse colony was established in 1970 by inoculation of a BALB/c female with MTV-S isolated from a C3H mammary tumour (kindly provided by Dr. Philomena Hageman, Netherlands Cancer Institute, Amsterdam). The descendants of this female had been sib-mated in our laboratory for 18 generations at the beginning of this investigation. The virus proved to be continuously transmitted to all females tested. The tumour incidence in breeding females is 100%, with an average latency period of seven months. Skin grafting at the 21st inbred generation proved the infected subline, indicated as BALB/cfC3H, to be completely histocompatible with the parental BALB/c strain which was used as recipient in this study.

Two-month-old BALB/cfC3H males were killed by cervical dislocation; organs were taken immediately thereafter. Cell suspensions were prepared by pushing cells with a spatula through a nylon gauze while immersed in Hanks balanced salt solution (HBSS). Viable cells were then counted by trypan blue dye exclusion and the original suspension was diluted with HBSS to different cell concentrations ranging from 10^6 to 10^3 per ml.

For the preparation of cell suspensions from liver and kidney, the kidneys and livers were first perfused *in situ* with Hommes medium without EDTA but supplemented with 0·05% collagenase (Sigma, St. Louis, Missouri, U.S.A.) and 0·10% hyaluronidase (Sigma) [3], in order to remove blood cells. The cell preparations were immediately injected intraperitoneally into 6-week-old BALB/c females, which then were subjected to forced breeding.

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Each group consisted of 20 females per dilution. Three females were caged with one uninfected BALB/c male. The cages were inspected daily for litters; these were immediately removed. The mice were inspected for the presence of mammary tumours or other signs of disease twice a week. The experiment was terminated when the animals were one year of age, because uninfected BALB/c females start to produce mammary tumours at that age.

In one series of experiments, cells were irradiated with a Philips–Müller X-ray machine (300 kv, 10 mA, HVL 3 mm Cu) at a dose rate of 110 rad/min to give a total dose of 3000 rad and injected immediately thereafter.

Cell-free extracts were prepared by homogenizing freshly taken tissue in cold HBSS (10 ml/g of tissue) in a Sorvall omnimixer (19,000 rev/min) for 50 sec. The preparation was then spun in a Sorvall superspeed RC 2-B centrifuge for 20 min at 15,000 rev/min (4°C). The supernatant was withdrawn and filtered through a 0·45 μ Millipore disposable filter unit (Millipore SA, Bue, France). Dilutions were made in HBSS and then injected intraperitoneally into mice. The undiluted inoculum corresponded with approximately 10^7 cells/mouse. Each group consisted of 20 animals.

RESULTS

The mammary tumour incidences at 1 year of age for the experimental groups injected with different cell numbers from various organs are given in Table 1; lung, pancreas and brain were completely negative. Even when 10^7 cells from these tissues were injected, no mammary tumours were found in the recipients. Relatively large cell numbers were needed for liver, kidney and testis.

Irradiation did not completely destroy the oncogenic activity of cells from various organs, but the number of cells from hemopoietic organs needed to produce a tumour is approximately a hundredfold higher (Table 2). This procedure had little effect on kidneys and epididymis cells in this respect.

Cell-free extracts from kidney, testis, epididymis and salivary gland were the only ones active in the bioassays (Table 3). Although for mammary tumour induction relatively few intact cells were needed from hemopoietic

Table 1. *Mammary tumour-inducing activity of intact cells from BALB/cfC3H mice in forced bred BALB/c female mice*

Organ	Number of cells:	Tumour incidences (%) at 1 year of age			
		10^6	10^5	10^4	10^3
Thymus		100	65	75	70
Spleen		90	50	30	25
Bone marrow		75	90	60	30
Salivary gland		100	100	80	40
Brain		0	0	0	0
Lung		0	0	0	0
Liver		80	60	10	0
Pancreas		0	0	0	0
Kidney		75	50	20	10
Testis		90	100	40	5
Epididymis		100	100	60	55

Table 2. *Effect of irradiation* (3000 *rad*) *on mammary tumour-inducing activity of BALB/cfC3H cells*

Organ	Number of cells:	Tumour incidences (%) at 1 year of age			
		10^6	10^5	10^4	10^3
Thymus		80	35	5	0
Spleen		30	40	0	0
Bone marrow		40	5	0	0
Liver		0	0	0	0
Kidney		50	70	30	20
Epididymis		70	30	45	30

Table 3. Mammary tumour-inducing activity of cell-free extracts of organs from BALB/cfC3H mice

Organ	Dilutions:	Tumour incidence (%) at 1 year of age			
		undiluted	10^{-1}	10^{-2}	10^{-3}
Thymus		0	0	0	0
Spleen		0	0	0	0
Bone marrow		0	0	0	0
Salivary gland		40	45	10	0
Liver		0	0	0	0
Kidney		50	10	20	0
Testis		25	5	0	5
Epididymis		75	25	55	20

tissues, such as thymus, bone marrow or spleens, cell-free extracts from these tissues were completely negative in the induction of mammary tumours. Extracts from perfused liver also failed to show mammary tumour-inducing activity.

DISCUSSION

The finding of infectious virus in the epididymis of BALB/cfC3H mice is in accord with the demonstration of MTV-S in that organ of other high cancer strains [4–6]. Mature MTV virions (B-type particles [7]) have been detected in the salivary glands of wild mice [8]. The demonstration of infectious virus in salivary glands of exogenously infected mice is therefore not so surprising.

We failed to find a report on the presence of infectious virus or B-type particles in kidneys from high cancer strain mice, but Parks *et al.* [9] found a relatively high titer of MTV-specific antigens in kidneys of the C3H strain. Cultures of kidney cells from various strains after treatment with various compounds release B type particles [10–13]. They represent endogenous viruses, however. It would be interesting to determine whether kidneys from exogenously infected mice would release MTV-S spontaneously. It has been claimed that BALB/c kidney cells could be productively infected *in vitro* [11].

The presence of MTV-S in the testis is in agreement with a similar finding of Pogossiantz [14] in another mouse strain. However, the testis has been reported to be negative for MTV antigens [15]. Since Leydig cell tumours of MTV-infected mice produce intracytoplasmic A particles [16] which are thought to be the precursory stages of B-type particles [7, 17], it is possible that the virus will be replicated in only a limited number of cells in the testis. A relatively small quantity of MTV-S is needed for the induction of mammary tumours in BALB/c mice [18], but considerably larger amounts of viral antigens are required for detection by immunological methods.

No B-type particles have been found in the pancreas, liver and spleen [19], which explains the lack of infectivity of cell-free extracts of these organs. B-type particles have been found in an epididymoblastoma [20] as well as in a pulmonary carcinoma [21]. MTV will probably not be expressed as complete virion in nonneoplastic cells. MTV-specific antigens have been found in the lungs of C3H mice [9] and, in our laboratory, also in the lungs of BALB/cfC3H mice. The negative bioassays may be explained by only partial expression of the viral genome.

Most attention has been paid to the presence of MTV-S in hemopoietic tissues [18, 22]. Viral antigens can be detected in low amounts in the spleen [9, 23]. Intracytoplasmic A-type particles have been found in several hemopoietic tissues of RIII mice with mammary tumours [6] and in neoplasms of such tissues [2, 6, 24]. As in other studies, cell-free tumour-inducing activity could not be obtained from thymus, spleen and bone marrow, probably because no complete virions were produced [25]. The radiosensitivity of the cell-bound infectivity of hemopoietic cells in contrast to that of kidney or epididymis suggests that viable hemopoietic cells are needed for the transfer of exogenous MTV-S. The infectivity of kidney or epididymis cells is probably due to complete virions, which are highly radioresistant [26].

The oncogenic activity of liver cells might be due to the presence of hemopoietic cells. However, the cell separation procedure followed by us removes all cell types other than parenchymal cells [3].

According to Michalides *et al.* [27], the DNA of liver cells from mice exogenously infected with MTV-S lacks significant parts (approx.

25%) of the MTV proviral sequences found in mammary tumours of these mice. This would imply that no somatic provirus of MTV-S is present in the liver. The detected homology (75%) would represent endogenous MTV. It is possible, however, that, as in the testis, only a small percentage of cells is infected with MTV-S. This would not be detectable by molecular hybridization and would explain why approximately 100× more liver than thymus cells are needed for infection. The lack of infectivity of cell-free liver extracts indicates that a similar virogenic relationship as in the haemopoietic tissues exists in the liver.

Exogenous mammary tumour virus seems to have a relatively wide organ distribution. It is only partially expressed in several tissues. This illustrates the important role of epigenetic factors in the expression of MTV.

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IgG-, IgA- and IgM-Antibodies to Herpes Simplex Virus Type 2 in Sera from Patients with Cancer of the Uterine Cervix*

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Abstract—*A total of 60 sera from patients with cervical cancer, 60 sera from matched controls, and 22 sera from 13 patients with recent herpes genitalis were examined by the indirect fluorescent antibody method for the presence of IgG-, IgA- and IgM-antibodies to herpes simplex virus (HSV) type 2. IgG-antibody titres in the patients with cervical cancer were significantly higher than those found in the control group, while IgA- and IgM-antibodies were normal. IgG-, IgA- and IgM-antibodies were found to be elevated in patients with frequent recurrent HSV type 2 infections. The elevation of a single class of immunoglobulin to HSV type 2 infection is serologically unusual and distinguish the patients with cervical cancer not only from normal controls but also from patients with frequent recurrent or chronic genital herpes.*

INTRODUCTION

SEROEPIDEMIOLOGICAL studies of herpes simplex virus (HSV) type 2 and carcinoma of the cervix have almost consistently shown that women with cervical cancer possess antibodies to HSV type 2 more frequently and in higher titres than matched controls [1–3]. Because of the cross-reactivity of antibodies to HSV type 1 and type 2 and because of the high incidence of antibodies to HSV type 1 in the community in general, the identification of HSV type 2 antibodies in human sera is difficult [4].

The variety of serological techniques used for the comparative titration of antibodies to HSV type 1 and type 2 have not been concerned with the type of immunoglobulin reacting in the test. According to Nahmias and Roizman [1] IgA- and IgM-antibodies to HSV seem to persist in measurable titres in patients with recurrent HSV infections. In a recent study made in our laboratory [5] we found a significant rise in IgA-antibodies to HSV following recurrent HSV infection. Furthermore this IgA-antibody response was type specific, as patients with genital herpes lesions responded with a marked rise in IgA-antibodies to HSV type 2 with only a weak and transient rise of IgA-antibody titre to HSV type 1.

If one accepts the hypothesis that a chronic or frequently recurrent HSV type 2 cervicitis is the underlying stimulus leading to malignant transformation of the cervical epithelium, then such a condition might serologically be better clarified through an analysis of the proportions of IgG-, IgA- and IgM-antibodies to HSV type 2, rather than by measurement of the total neutralizing activity of HSV type 2 of a given serum.

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MATERIAL AND METHODS

Sera examined

Blood from 60 patients with cervical cancer was furnished by the Finsen Institute in Copenhagen. The staging of the malignancy followed the international classification of carcinoma of the uterine cervix. Blood was collected only

from newly diagnosed patients before treatment was started. Blood samples from 60 normal controls were supplied by the blood bank at Rigshospitalet in Copenhagen and from the geriatric unit at "De Gamles By" (a major home for old people in Copenhagen). Twenty-two blood samples from 13 patients with recent herpes genitalis (virologically confirmed HSV type 2 infections) were included as a special control group.

The cases and normal controls were classified into five socio-economic classes according to the criteria used by the Danish National Institute of Social Research [6]. In the analysis of the results of the present study the subjects in social classes 1, 2 and 3 were placed in one group.

Measurement of HSV type 2 IgG-, IgA- and IgM-antibodies by indirect immunofluorescence

(1) *Antigen.* Confluent monolayers of VERO-cells maintained in Eagle's MEM in Earle's BSS and 2% foetal bovine serum were infected with the MS strain of HSV type 2 (ATCC lot: 1-D) at a multiplicity of 2 and harvested after 18–20 hr by shaking the cells off the glass surface. The cells were further dispersed by pipetting and washed three times with PBS. Smears of infected cells were made by placing one drop of the cell suspension on each of eight spots left clear on PTFE Plasti-Brand-coated microscope slides. After air drying, the cells were fixed in acetone for 15 min at room temperature. Slides were stored at −20°C. Control smears of uninfected VERO-cells were prepared in the same way, the cells being scraped off the glass surface.

(2) *Staining procedure.* Patients' sera were titrated for the presence of HSV type 2 IgG-, IgA- and IgM-antibodies by the indirect fluorescent antibody staining. Two-fold dilutions of the sera were prepared and drops of each dilution were applied on the smears of HSV type 2 infected VERO-cells. Control titrations of sera on uninfected cells were run in parallel. The slides were incubated at 37°C in a moist chamber for one hour when titrating IgG and IgA, and for two hours in the case of IgM. After incubation, the slides were thoroughly washed in PBS for ten minutes and for another ten minutes in deionized water by magnetic stirring.

Fluorescein-labelled rabbit immunoglobulins to human IgG, IgA and IgM (obtained from Dako Immunoglobulins A/S, Denmark) were used. The working dilutions of these conjugates were 1/20 ,1/15, and 1/15 respectively, and no fluorescence was seen when conjugates were used alone. The same batch of each antiglobulin was used throughout the study.

Air-dried smears were covered with one drop of the diluted conjugate. Slides were incubated at 37°C for 30 min in a moist chamber and then washed in PBS and water, as described before. After air drying, the slides were mounted in 10% elvanol/Tris–HCl buffer, pH 8.

A Leitz–Wetzlar microscope equipped with a halogen quartz lamp (Phillips, 12 V), an interference filter (Werner Olsen, Copenhagen), and a 515 nm barrier filter, was used to read the slides.

The serum fluorescent antibody titre was expressed as the reciprocal of the highest serum dilution giving bright green fluorescence of at least 50% of the infected cells.

The neutralization data used in this paper were extracted from the kinetic neutralization test performed on the same sera in a previous study [3].

In the calculation of the arithmetic mean and standard deviation of the antibody titres, titres $\leqq 8$ for IgG and IgA and $\leqq 4$ for IgM, were considered 0. Titres $\geqq 2048$ were considered 2048. The distribution of antibody titres in the cancer group, normal control group and special control group was compared by the chi square test. Spearman's rank correlation coefficient analysis was used in the statistical analysis of the correlation between fluorescent IgG-, IgA- and IgM-titres and neutralizing activity of the same sera.

RESULTS

Age distribution and socio-economic status of the material

The age distribution of the patients with cervical cancer and the controls was almost identical (Table 1). The distribution of the cervical cancer patients did not differ from the distribution found in the control group with respect to socio-economic classes (Table 2).

Table 1. Age distribution of patients with cervical cancer and controls

Age (yr)	Cervical cancer	Controls
30–39	6	10
40–49	16	20
50–59	20	19
60–69	14	8
70–79	4	3
Total	60	60

Table 2. Distribution of patients with cervical cancer and controls according to socio-economic status

Socio-economic class	Cervical cancer	Controls
1, 2, 3	11	11
4	36	37
5	13	12
Total	60	60

The majority of women in both groups belonged in social group 4 (lower middle class).

Antibody titres in general

The mean fluorescent antibody titres (MAT) of IgG, IgA and IgM to HSV type 2 are shown in Table 3. The mean-IgG titre was higher in the cancer group as compared with the normal control group. The different distribution of IgG-titres in the two groups was found to be statistically significant, $0{\cdot}02 > P > 0{\cdot}01$, while no significant differences were found in the distribution of IgA- and IgM-titres between the cancer and normal control groups.

Table 3. IgG, IgA and IgM mean fluorescent antibody titre (MAT) to HSV type 2 in sera from 60 patients with cervical cancer, 60 normal controls, and 22 sera from 13 patients with recent herpes genitalis

	Cervical cancer	Normal controls	Herpes genitalis
IgG	502 ± 160	348 ± 158	522 ± 110
IgA	33 ± 12	27 ± 15	62 ± 22
IgM	22 ± 13	17 ± 10	42 ± 12

Statistical comparison between the cancer group and the group of patients with genital herpes showed no differences in the distribution of IgG-titres. IgA- and IgM-titres, however, were significantly higher in the patients with herpes genitalis than in the patients with cervical cancer.

Antibody titres in relation to age and socio-economic status

The youngest age groups of both cancer patients and controls had higher IgA antibody titres than the rest of the material, but the difference was not significant. Neither IgG-titres, nor IgM-titres to HSV type 2 had any relation to age.

Low socio-economic status could be correlated to high titres to HSV type 2 in all three immunoglobulin classes in the normal control group. In the cancer group there was no correlation between antibody titres and socio-economic status.

Antibody titres in relation to stages of malignancy

Table 4 shows that the mean titre of IgG-antibodies increased with increasing development of the malignant process, while IgA- and IgM-antibody titre had no relation to the severity of the disease.

Table 4. IgG, IgA and IgM mean fluorescent antibody titre (MAT) to HSV type 2 in sera from 60 patients with cervical cancer in relation to stages of the malignancy

	Number of patients	IgG	IgA	IgM
Stage 1	27	382 ± 150	27 ± 12	20 ± 14
Stage 2	20	460 ± 160	32 ± 11	24 ± 11
Stage 3	13	640 ± 190	27 ± 12	17 ± 14

Comparison of HSV type 2 neutralizing activity and fluorescent IgG-, IgA- and IgM-antibody titres to HSV type 2 in the individual sera

The fluorescent IgG-antibody titres and the neutralizing activity against HSV type 2 showed a high degree of correlation. Rank correlation analysis gave a correlation coefficient of 0·59, corresponding to a T value of 4·44 or $P \geqq 0{\cdot}001$. No correlation was found between IgA- or IgM-antibody titre and neutralization. Sera from the cancer group and the normal control group showed the same pattern.

DISCUSSION

Indirect fluorescent assay of different classes of immunoglobulins performed on whole sera raises the question as to whether the observed titres represent true changes in the amount of different immunoglobulins reacting with HSV antigens, or merely reflect the result of competition between the different immunoglobulins for the same antigenic sites. This possibility was tested in a previous study [5] by fractionation of the immunoglobulins on sucrose gradient and by analysis of the kinetics of IgG- and IgA-antibodies in serial blood samples from the same individual. We reached the conclusion that competition between IgG- and IgM-antibodies did not occur, and that competition between IgG- and IgA-antibodies, although likely, could not be demonstrated.

Raised titres of IgA- and IgM-antibodies in relation to recent or chronic infection with HSV and other viruses have been demonstrated in a number of studies [1, 5, 7–9].

Aurelian and co-workers [10] have demonstrated an IgM-response in cervical cancer patients directed against an HSV-coded membrane-associated antigen (Ag-4). The reason that we failed to demonstrate a rise in IgM-titre by the indirect fluorescent antibody method could be that the VERO-cells infected for 24 hr and used as indicator cells did not produce Ag-4.

The cancer patients were found to have an IgG-antibody titre to HSV type 2 which was not only significantly higher than that of the controls, but reached the same level as found in patients with recent genital infection with HSV type 2. This elevated fluorescent IgG-antibody titre could be correlated with the higher neutralizing activity of HSV type 2 found in the same cancer sera as compared with the controls. This means that the elevated IgG-antibodies must be directed against structural antigens in the viral envelope [11, 12] and do not necessarily represent antibodies to non-structural or tumor-associated HSV antigens.

Vos and co-workers [13] have shown cervical tumor tissue to contain large amounts of immunoglobulins, predominantly IgG. These antibodies had a marked lymphocytotoxic activity against a known lymphocyte panel used by routine for HL-A typing. The authors suggest that the formation of such partly autoimmune antibodies might impair the function of the host's own cell-mediated immune system and in turn lead to an unrestricted synthesis of immunoglobulin. This hypothesis is supported by our finding that increasing titres of IgG against HSV type 2 infected cells were found in patients with increasing spread of the malignant process and, as found by others [14], that titres of neutralizing antibodies to HSV type 2 frequently increased during prolonged disease.

However, Sprecher-Goldberger and co-workers [15] found that blood cells from patients with cervical cancer were stimulated with HSV type 2 and HSV type 2 transformed cells above the level found in healthy women. This effect could even be demonstrated in cancer patients with a decreased cell-mediated responsiveness in general, as measured by phytohemagglutinin stimulation.

The answer to the pertinent question as to whether the increased antibody titres against HSV type 2 found in patients with cervical cancer are caused by a disturbance of the immunological system brought about by the malignant process, or are the result of a stimulation of the humoral response to HSV specified antigens present on the tumor cells, must await further studies.

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Establishment and Characterization of the Cell-Line of a Human Edonmetrial Adenoacanthoma

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Abstract—*A new cell-line of a human endometrial adenoacanthoma, designated as HEC–6, has been established and propagates well with a permanent stability. The population doubling time of the line is calculated as 52 hr. The* in vitro *morphology reveals the features of a well-differentiated adenocarcinoma such as a jig-saw puzzle-like arrangement and the formation of an acinous cell-free space in the monolayer culture. Each cell consists of a round atypical nucleus with 2 or 3 marked nucleoli and a vacuolated cytoplasm. A piling-up tendency is a prominent feature and forms an obvious palisade-like or glandular structure. The karyotype of the stem cell is a pseudo-diploid in which variant chromosomes occur at random in various groups. When transplanted into a hamster cheek pouch, line HEC–6 demonstrated an adenocarcinoma in addition to a stratified epithelial component. The original characteristics of an adenoacanthoma of the corpus uteri have been maintained in the culture system.*

INTRODUCTION

THE ENDOMETRIUM, a target of sex steroid hormones, undergoes a cyclic alteration by the effects of the hormones. It is important to know if the endometrial gland still preserves the ability as a hormone target even after its maligant transformation. The various characteristics of human endometrial carcinoma should include the relation to hormones, however, because of the lack of a suitable experimental tool they have yet to be identified in detail. An *in vitro* cellular system might contribute to these purposes, supplying a convenient research method. We have established line HEC–1 originating from an adenocarcinoma of the corpus uteri and identified various features of the tumour using this *in vitro* experimental system [1–3].

The present paper reports the establishment of the new corpus tumour cell-line, its morphology, cytogenetic features and heterotransplantability as well as tissue reproducibility.

MATERIAL AND METHODS

Case history of the culture material

The fresh tumour material was obtained from a 48-year-old patient with carcinoma of the body of the uterus. This nulliparous patient has been well with regular menstrual cycle until she first complained of intermittent bloody discharge for 2 months duration. She used to live in Hiroshima and was 25 when an atomic bomb exploded there during World War II. An endometrial curettage showed an adenoacanthoma of the endometrium and the portion of a squamous epithelium was rather atypical in shape (Fig. 1). The patient was treated by total hysterectomy, bilateral salpingo-oophorectomy and pelvic lymphadenectomy on July 24, 1970. The uterus, the size of a small goose egg, showed a circumscribed tumour on the fundus, bulging into the cavity. The specimen adjacent to the cultured material revealed a well-differentiated adenocarcinoma.

Culture method

A monolayer culture method was used. The tumour material from the excised uterus was first washed twice in a phosphate buffered solution (Ca^{2+} and Mg^{2+} free) and minced into fragments with fine scissors and ventilated in 0·25% trypsin solution. The fluid and the minced tumour were stirred slowly by a magnetic stirrer (500 rev/min) for 15 min.

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The trypsin solution was changed 4 times. Then the turbid fluid in which the explant materials were dispersed into a single-cell suspension was aspirated and centrifuged twice after mixing with a culture medium. Subsequently, the sediment was resuspended in medium and placed on a stationary culture in 5% carbon dioxide and 95% air or, occasionally, in a closed vessel. Subcultures were made after treating them with solution of 0·1% trypsin and 0·02% ethylendiamine tetraacetate (EDTA).

Growth medium

Tissue culture medium No. 199 supplemented with 15% calf serum was used, containing penicillin in 100 U/ml and streptomycin in 100 μg/ml.

Morphologic and chromosome analyses

Approximately 5×10^4 to 1×10^5 cells were inoculated on a square test tube (40 by 17 by 8 mm in size; MA–8 Miharu) containing a cover glass, 32 by 12 mm, and cultured for 3–7 days.

The living cells were observed with an inverted phase-contrast microscope. Cells on a cover slip were also fixed with absolute methanol and stained with 10% Giemsa solution. Periodic Acid Schiff (PAS) and Alcian blue stains were made using twice the normal staining time.

For chromosome analysis, the medium was exchanged to one containing Colcemid, 5×10^{-7} M and the cells were incubated for 1 to 2 hr at 37°C to arrest those in metaphase. The cover slip with the cells was treated by a hypotonic solution, placed carefully in one minute in 0·7% and 0·5% sodium chloride solution and then in 0·2% sodium chloride solution for 20 to 40 min. Then the cells were fixed by passing through 1, 10 and 100% Carnoy solution (3:1 aceto-alcohol) for 1 to 2 min and finally for 15 min in the fresh 100% Carnoy solution. The specimens were air dried and stained with 10% Giemsa solution (pH 6·8). Three hundred and sixteen of the metaphase cells were observed to calculate the modal ploidy and 25 of the modal range were counted exactly to determine the chromosomal number. The karyotype of the cells with the modal of chromosomes was carefully analysed in accordance with Denver nomenclature.

Cell cultivation for growth curve

After being dispersed by a mixed solution of 0·1% trypsin and 0·02% EDTA, the cell suspension containing 2×10^5 cells was inoculated into plastic Petri dishes (60 mm in diameter; Lux Plastics) and grown as replicate cultures [4] in a humidified atmosphere containing 5% CO_2 and 95% air. Every 2 days after the subculture, two dishes were harvested to count the number of cells. The media in the remaining dishes was changed. The number of cells in each dish was counted 4 times, using a Burker–Türk counter plate. The mean was calculated for each dish and for every harvest. The data was plotted as a semilogarithmic graph.

Transplantation into a hamster

A female hamster, 4 weeks old and weighing about 50 grams, was used for the experiment. The cell cultures were dispersed into a suspension of single cells by the trypsin-EDTA solution, and then were washed with culture media and centrifuged twice. The cells, 42·0 or $75·0 \times 10^5$ in number, were injected into the submucosa of a hamster cheek pouch by a syringe with a No. 22 gauge needle. The hamster was anaesthetised by 2 mg of sodium pentobarbital intraperitoneally, and also immunosuppressed by giving cortisone acetate 2·5 mg per 50 g of body weight once every 4 days, starting on the day of transplanation.

RESULTS

Culture course to the establishment

The cells were attached well on the bottom of a culture bottle on the first day of the primary culture forming multiple sheets. The culture was apparently successful from the beginning, in contrast to other unsuccessful attempts. The primary culture was grown for 18 days before the first subculture. Since then the cells have been subcultured every 7–14 days, being diluted to three- or four-fold on each passage.

The cell proliferation has been maintained except for 4 accidental growth-suppressive periods. It seemed, however, that they happened to be due to inappropriate calf serum, or inappropriate incubation conditions, rather than a characteristic feature of these cancer cells in culture. The line was designated as HEC–6 (human endometrial cancer—6).

When confluent, HEC–6 cells pile up easily from a monolayer sheet. However, if the cells are not subcultured the sheet often will shed cells and debris into the medium. On the other hand the cells are not easy to trypsinize into single cell suspension, especially in logarithmic growth periods but become easy when confluent.

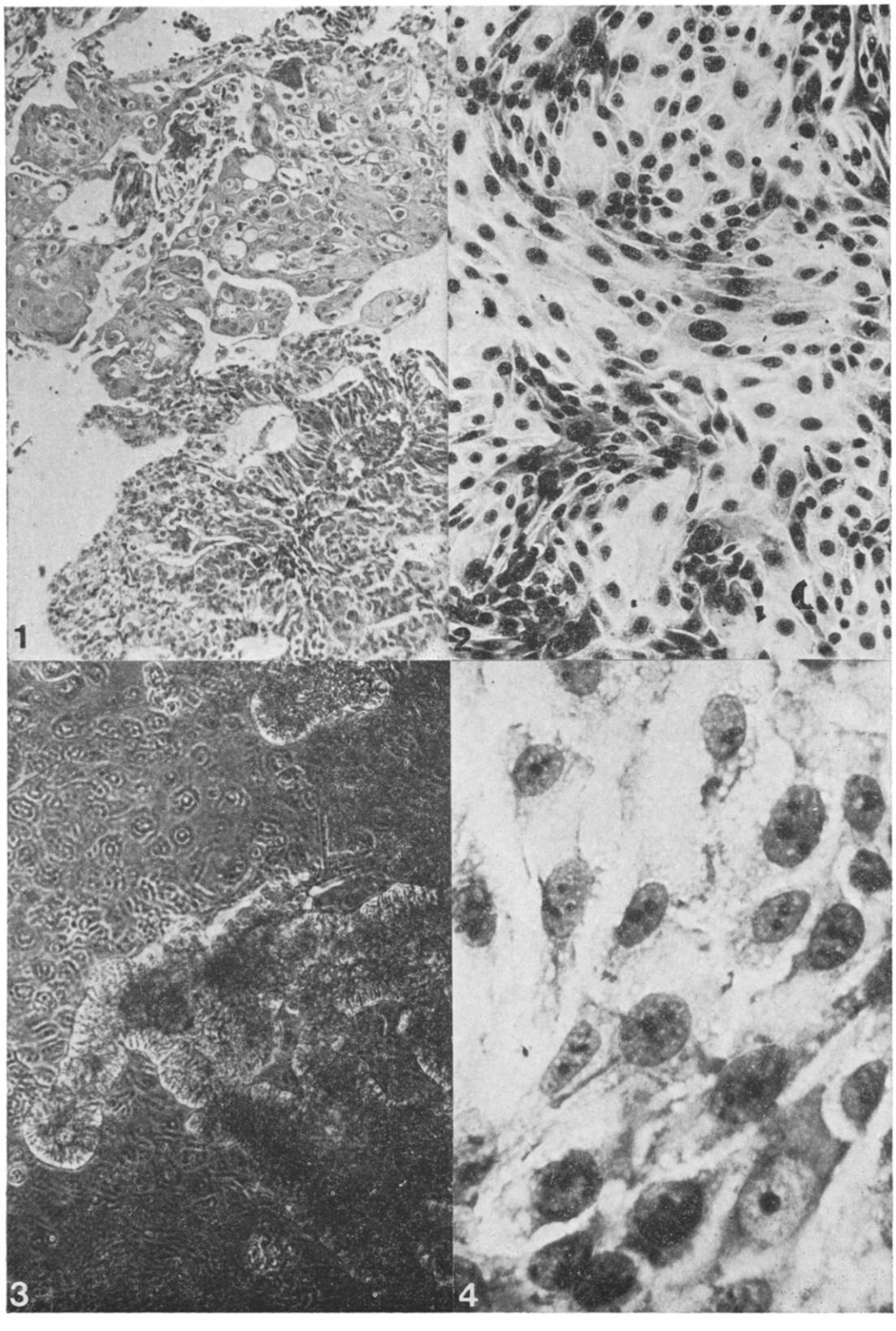

Fig. 1. *Histology of the original tumor showing adenoacanthoma. Hematoxylin and Eosin (H–E) stain.* × 150.

Fig. 2. *The monolayer-cultured HEC–6 cells reveal a jig-saw puzzle-like or a wheeled pattern. Giemsa stain.* × 150.

Fig. 3. *The piling-up growth of line HEC–6. Note palisade- or gland-like arrangement. Phase-contrast microscopy.* × 150.

Fig. 4. *Magnified view of the line showing the cells with anisonucleosis and marked nucleoli. The cytoplasms of which borders are obscure preserve vacuoles. Giemsa stain.* × 600.

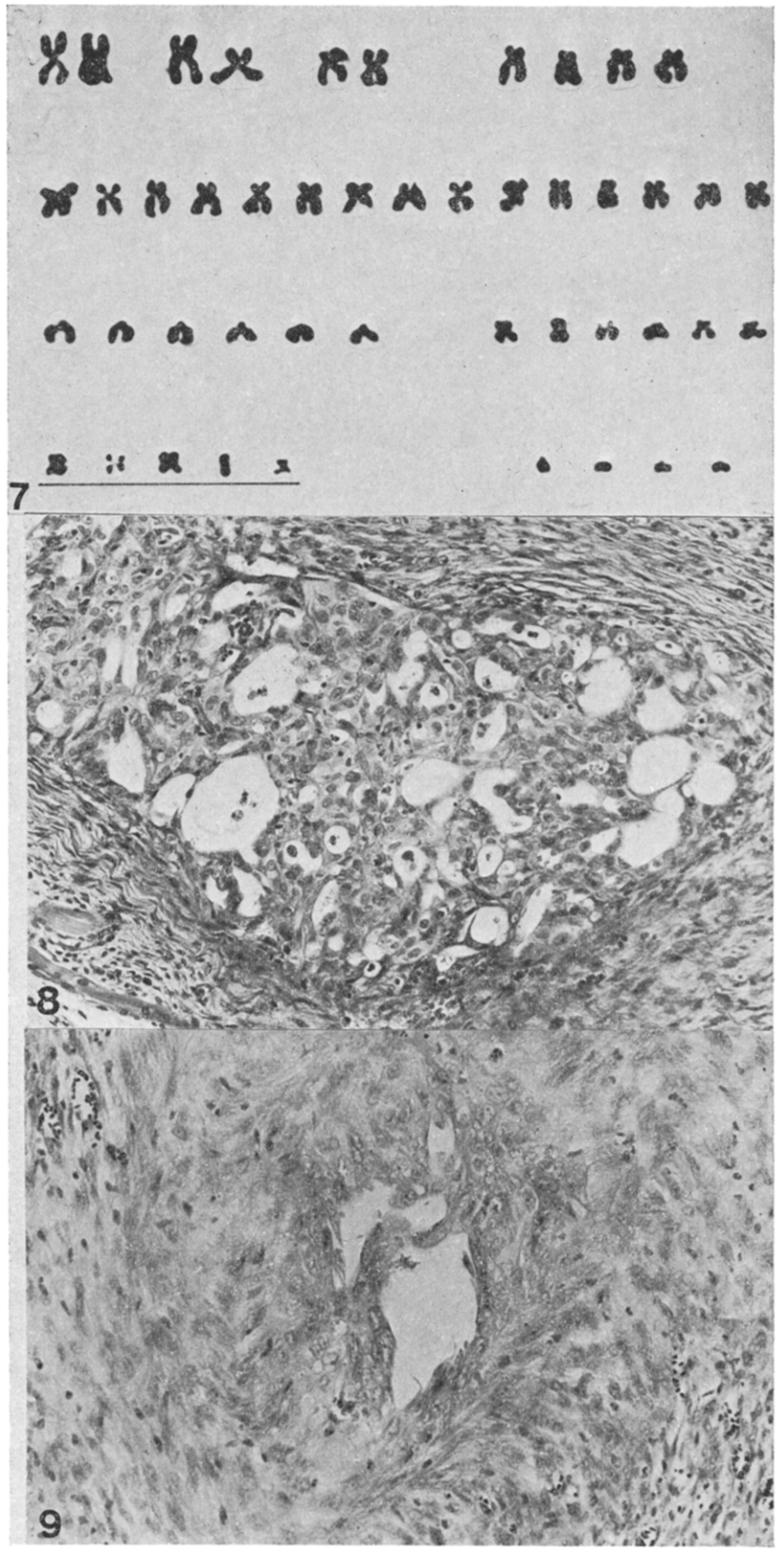

Fig. 7. A karyotype of the stem cell showing no apparent structural aberrations except numerical alteration in group C and F.

Fig. 8. Histology of the tumor when transplanted into a hamster revealing adenocarcinoma. H-E stain. × 150.

Fig. 9. The reconstruction of a stratified pavement epithelium is also demonstrated on the other view. H-E stain. × 150.

Morphology

Since the primary culture the cells have remained homogenous and polygonal shaped. No fibroblastic cells have been seen. The arrangement of the actively growing cell sheet appears stream-shaped in polarity, showing a jig-saw puzzle-like or a wheeled pattern (Fig. 2). When the growth progresses HEC–6 cells grow in multilayers and seemingly reconstruct an original structure obviously forming a palisade-like formation (Fig. 3). Conversely, when the cells grow in a monolayer sheet they proliferate most readily around the cell-free spaces. This seems to be an expression of acinous formation.

The cells of the primary culture were classified as malignant columnar cells according to the criteria of a diagnostic exfoliative cytology. They had hyperchromatic nuclei, of varying size, irregular chromatin distribution and one or more large nucleoli. The plasma membrane was ill defined. There was abundant cytoplasm containing many vacuoles (Fig. 4). PAS-positive granules, that were removed by digestion with saliva, were present in the cytoplasm. Alcian blue staining was negative. These morphologic features have been retained by the culture cells. However, the PAS-positive granules disappeared at 30th generation until then they had been a constant feature of the cytoplasm.

Growth kinetics

The growth curve of HEC–6 cells was examined at 37th passage generation (Fig. 5). The growth showed lag phase, lasting 5 days, followed by a logarithmic growth phase until the 11th day. Subsequently, the growing rate declined to enter a stationary phase. The population doubling time of the line HEC–6 during the logarithmic growth phase was 52 hours.

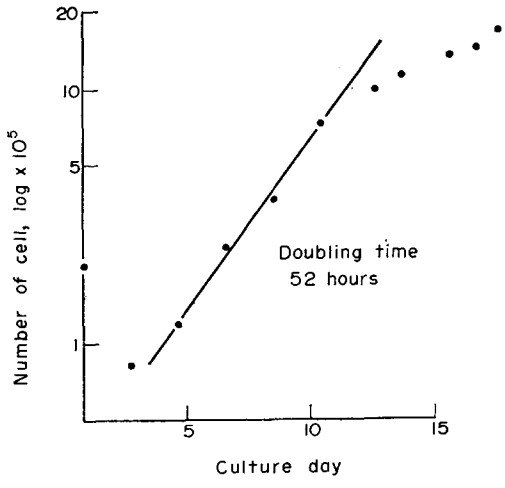

Fig. 5. Growth curve of HEC–6 at 37th generation. The population doubling time is calculated as 52 hours.

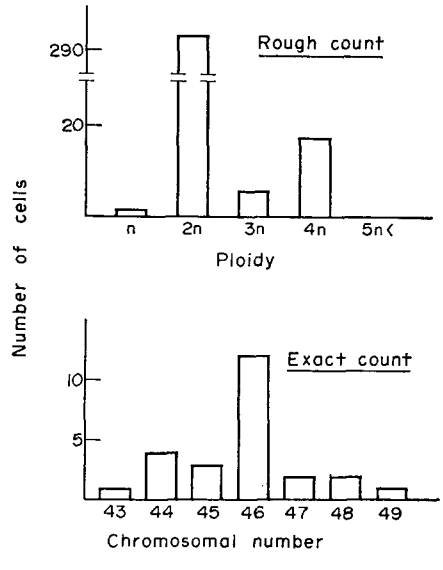

Fig. 6. Distribution of the chromosomal number at the primary culture. The majority of 316 metaphase cells appeared to be on a diploid range by a rough-counting (above) and the exact counting of 25 cells on the diploid mode showed the peak on 46 in number (below).

Chromosome constitution

The chromosome analysis was performed on the primary culture. The modal ploidy of 316 metaphase cells appeared to be obviously diploid by approximate counting. Very few cells deviated from the diploid mode. The exact counting of chromosome number showed the modal peak to be 46 (Fig. 6). The karyotype of the mode was analysed. There was no specific chromosome aberration or marker chromosome (Fig. 7), but irregular increase or decrease of chromosome number in each group was noted. Chromosomes exhibiting slight morphological aberrations were present in almost every group (Table 1). From these findings, it is deduced that the HEC–6 line arose from a pseudo-diploid stem cell.

Heterotransplantation

The HEC–6 cells at 11th, 16th and 26th generation respectively were transplanted into the submucosa of a hamster cheek pouch and produced a definite tumour. Histologically in some areas these tumours were typical of adenocarcinoma (Fig. 8). In another field, there appeared to be no gland formation or cells with cytoplasm of a secretory type, but there was construction of a stratified epithelium which was considered as a squamous meta-

Table 1. *Karyotype analysis of HEC–6 on* 46 *chromosomal number at the primary culture. Variant chromosomes* (*) *which show minimum morphological aberrations are scattered at random in various groups. The group constitutions in number are altered*

No.	Sketch No.	Chro. No.	A1	A2	A3	B	C	D	E	F	G
1	9	46	2	3	2*	4	14	7	4	6	4
2	10	46	2	2+1*	1*	2+3*	15	5	6	4	4+1*
3	11	46	3	2*	2+1*	4	14	6	6	4	4
4	19	46	2	2+1*	2	4	13	5+1*	6	4	5+1*
5	20	46	2	2	2	4	13	4+1*	7+2*	4	5
6	24	46	2	2	2	4	15	6	6	5	4
7	25	46	2	2	1+1*	3+1*	17	6	4+1*	4	4
8	4A	46	1	2	1+1*	3	19	5	6	4	4

*Variant chromosome.

morphosis (Fig. 9). The findings coincide well with those of the original adenoacanthoma.

DISCUSSION

One of the authors was the first to report the established line, HEC–1, of human corpus tumour in 1972, originating from a moderately differentiated adenocarcinoma [1, 2], and mechanism of the progesterone therapy upon endometrial carcinoma was studied by using this line. The suppressive effect on the growth kinetics and the morphological alterations were reported elsewhere [3]. The characteristic features of line HEC–6 such as the cellular arrangement, diploid modal chromosomal number and heterotransplantability are similar to the HEC–1 line. Conversely, there is the detailed histology of the reconstructed tumour grown as a xenograft and absence of a marker chromosome.

Adenoacanthoma of the uterine corpus may be defined as a lesion composed of anaplastic glandular element in addition to which there is an epidermoid epithelial component. The latter is usually benign [5]. The histogenesis of the squamous-metaplastic change has been debated. The indifferent or reserve cell, lying between the cylindrical cells and the basement membrane, may give rise to stratified squamous epithelium in response to a variety of stimuli. This might possibly arise in malignant as well as benign endometrial glands. The carcinoma cells may have keen capable of differentiating into squamous epithelium; rather than squamous metaplasia of the normal endometrium, as the result of an inflammatory response from the adjacent carcinoma. The xenograft of the HEC–6 cells seems to give an answer to this question, since no normal endometrial components which may transform to a squamous epithelium are present in the graft site. The cultured cell differentiates both adenomatous and squamous elements in an appropriate environment *in vivo*. The individual cell characteristics determine the malignancy of the lesion.

The stem cell of line HEC–6 is a pseudo-diploid. Likewise, various investigators [6–12] have reported, using squash or flame-dry methods, that endometrial cancer has a diploid or near diploid mode. Tseng and Jones [13], and Katayama and Jones [10] found an entirely normal karyotype in endometrial tumours including an adenoacanthoma which has never been reported in other malignant neoplasms. However, Wagner *et al.* [14] observed a wide variety of aneuploidy states based on the distribution of nuclear DNA content, and Takemura [6] found polyploid modes in two cases. Katayama and Jones [10] also stated that in an anaplastic adeno-carcinoma and an adenosquamous carcinoma more than half of the cells had hyperdiploid chromosomes. On the other hand, Atkin *et al.* [15] demonstrated all of the 7 well-differentiated corpus tumours were diploid or hypodiploid, whereas there were relatively fewer corpus tumours in the tetraploid region and none with DNA values above tetraploid. There may be evidence of a correlation between the histologic grading of malignancy and the extent of chromosome aberrations in the carcinoma of the endometrium, although the majority of the reported cases as well as HEC–6 gather to a diploid range.

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The Role of T and Suppressor Cells in MTV-Directed Cellular Immunity*

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Abstract—*The lymphocyte population from mice immunized with mammary tumour virus (MTV) which responds* in vitro *with blastogenesis in the presence of MTV, proves to be T cells. In mice bearing spontaneous MTV-induced mammary tumours, this T cell reactivity was very often depressed. The depression was partly due to the presence of suppressor cells which have adherent properties. These suppressor cells appear early during tumour growth and exert their greatest influence when the tumour weighs* 0·5–1·5 *g. In animals bearing larger tumours less suppressor cells were present and the hyporeactivity in the lymphocyte stimulation test must also be due to other factors.*

INTRODUCTION

WHEN incubated with mammary tumour virus (MTV) leukocytes of mice with virally induced mammary tumours show virus-specific blastogenesis in the leukocyte stimulation (LS) test (preceding paper).

Blair and Lane [1] state that MTV-specific cytotoxicity is a property of T cells and null cells which are recruited by an MTV specific factor produced by B cells. These null cells are found in the cell fraction that does not adhere to nylon wool, but they cannot be killed by antitheta serum and complement.

However, despite these immune responses, primary mammary tumours show progressive growth. It is reported by Blair and Lane [1] that, as the tumours of the female mice increase tn size, the null cell cytotoxic activity is no longer detectable, but they do not mention a loss of T cell dependent cytotoxicity in the microcytotoxicity (MC) test. MTV-specific leukocyte stimulation and Leukocyte Adherence Inhibition (LAI) in mice decreased to almost zero when the tumour reached a weight of about 2 g (preceding paper). In human breast cancer, LAI reactivity decreased with increasing tumour burden [2].

It has been shown that some *in vitro* lymphocyte functions, as for instance stimulation by plant lectins, can be inhibited by suppressor cells present in the spleen of animals bearing progressively growing tumours: Glaser *et al.* [3] demonstrated this in animals with Gross Leukemia Virus induced tumours. The same phenomenon was also shown in the Moloney Sarcoma Virus (MSV) system [4, 5]. The suppressor cells were thought to be macrophages.

To further study the mechanisms of MTV-directed cellular immunity in mice bearing primary mammary tumours, we again used the LS test. Evidence is presented that T cells are most likely responsible for the reactivity observed, and that the decline in blastogenesis with increasing tumour weight is caused, among others, by the presence of suppressor cells which have adherent properties.

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MATERIAL AND METHODS

BALB/cfC3H mice carrying MTV-S and GR, which spontaneously releases the endogenous MTV-P, were used. Tumours were induced in these mice by forced breeding. Male mice of 12 weeks of age were used as positive controls, they were immunized i.p. with 1 μg disrupted alum precipitated virus and killed 5 days later. Male mice not older than 10 weeks served as normal controls, because they do not release endogenous MTV at that age. All mice were killed by cervical dislocation.

Virus

The standard strain of mouse mammary tumour virus (MTV-S) was isolated from

BALB/cfC3H mammary tumours, as described in the preceding paper. Again, virus content was estimated on the basis of the amount of protein, as measured by the Folin method. Before use as antigen, the virus was disrupted by repeated freezing and thawing.

LS test

This test was carried out as described in the preceding paper. Blast formation was estimated by the incorporation of 2-^{14}C-thymidine. In all experiments 10^{-4} mg MTV/ml was added; purified RLV (10^{-4} mg/ml) was used as a control throughout. The cultures were run in triplicate. Statistical significance was established by means of the two-tailed Student's *t*-test. Per cent stimulation was calculated according to the formula: $a-b/b \times 100\%$ in which a is the average counts/min (cpm) of the cultures incubated with MTV and b is that in the cultures incubated with RLV. To detect whether the spleen of animals bearing tumours of different sizes contained suppressor cells, individual animals were tested: A T-cell-enriched cell fraction was made (see below) and $0{\cdot}5 \times 10^6$ spleen cells derived from the same animal in 0·2 ml medium supplemented with 20% foetal calf serum were added to each culture. Cultures to which normal spleen cells in the same concentration were added served as controls. All cultures were incubated with MTV. Per cent inhibition was calculated by: $(1-c/d) \times 100\%$, in which d is the average cpm of the cultures containing T-cell-enriched populations to which spleen cells containing suppressor cells were added and d is that in the cultures to which normal spleen cells in the same concentration were added.

Separation of cell subpopulations

B-cell-enriched fractions were obtained by incubating 4×10^6 macrophage-depleted spleen cells with mouse antitheta serum and complement for 1 hr. The anti theta serum was made by injecting 2×10^7 thymocytes derived from C3H mice intraperitoneally into AKR mice. This was done six times with intervals of 14 days. Then the AKR mice were bled; the resulting ant tiheta serum killed 70% of C3H thymocytes up to a dilution of 1/16. As a source of complement guinea pig serum absorbed with agarose and stored in liquid nitrogen was used. The complement was active up to a dilution of 1/8. After treatment, the cells were washed and used for the LS test.

T-cell-enriched cell populations were obtained by adherence on a nylon wool column [6]. After elution about 20% of the original cell population was recovered. Of the eluted cell population, 60–70% could be killed with antitheta serum and complement, whereas 10% were shown to be Ig-positive B cells in direct membrane fluorescence studies.

Adherent cells were removed by incubation of spleen cells (4×10^6/ml) in Falcon tissue culture flasks (25 cm^2) during 2–3 hr. When adherent cells in combination with T-cell-enriched populations were tested, the adherent cells present in $2{\cdot}5 \times 10^6$ spleen cells were allowed to adhere in the tubes in which the LS-test was performed; the supernatant was then removed and the adherent cells were washed three times with medium, after which T-cell-enriched cell fractions were added. The approximate number of adherent cells was estimated by subtracting the number of cells that did not adhere from the original concentration.

Membrane fluorescence

For the detection of viral antigen that could be attached to the cells, adherent cells were grown in microtitre plates as is described by Sorg [7]. They were washed three times and then incubated for 1 hr with goat polyvalent anti-MTV-S serum at 37°C in a humidified atmosphere. After three washings in PBS, the cells were treated with fluorescein conjugated antigoat Ig serum (Nordic, Tilburg, The Netherlands) which was diluted 1:20 in PBS and incubated for 30 min. They were again washed three times prior to observation.

Antibody attached to the cells was determined by treatment with fluorescein conjugated antimouse Ig serum, which was also used in a 1:20 dilution.

RESULTS

MTV-specific blast forming cells

In every experiment, the spleen cells used were derived from at least 3 positive control animals. If lymphocytes, T-cell-enriched and B-cell-enriched cell populations prepared from the same pool of spleen cells were compared, the T-cell-enriched fraction showed the highest stimulation as compared to the RLV-control, whereas the degree of blastogenesis observed with the same number of cells of the B-cell-enriched cell population was not noteworthy (Table 1). Even if stimulation of the spleen cells was not significant, significant stimulation emerged when T-cell-enriched cell fractions were used.

Stimulation of the T-cell-enriched fraction was not increased if adherent cells were added

Table 1. *Proliferation of different cell populations of positive control animals prepared from the same pool of spleen cells*

Mouse strain	Cell population	Experiment 1 Counts/min ± SE* in the presence of RLV	Experiment 1 Counts/min ± SE in the Presence of MTV	Experiment 1 Per cent stimulation	Exp. 2 Per cent stimulation	Exp. 3 Per cent stimulation
BALB/cfC3H	Lymphocytes	2·237 ± 712	3·226 ± 233	44‡	121†	87†
	T-cell-enriched	1·652 ± 318	3·595 ± 184	117†	246†	154†
	B-cell-enriched	1·086 ± 312	1·224 ± 218	13‡	29‡	0‡
GR						
	Lymphocytes	5·161 ± 561	15·889 ± 249	211†	53‡	74†
	T-cell-enriched	1·721 ± 203	7·273 ± 567	322†	96†	137†
	B-cell-enriched	2·640 ± 416	2·841 ± 268	17‡	4‡	2‡

*SE: standard error of triplicate cultures.
† $P < 0.010$.
‡ Not significant.

as compared to T cells to which adherent cells derived from normal animals were added (Table 2).

Suppressor cells

In both GR and BALB/cfC3H mice bearing large MTV-induced tumours (2 g or larger), it was repeatedly observed that the T cell response was markedly depressed if adherent cells derived from the same animals were added, as compared to cultures to which adherent cells derived from normal animals were added (Table 3). When different concentrations of adherent cells derived from mice bearing large tumours were added to T-cell-enriched fractions derived from mice bearing very small tumours, blast formation in the presence of MTV decreased gradually. This was not the case when adherent cells from normal animals were added (Fig. 1).

To determine whether the cellular hyporeactivity seen at a tumour weight exceeding 2 g (preceding paper) can be attributed to suppressor cells, individual BALB/cfC3H mice bearing tumours of varying sizes were tested for the presence of such cells. To T-cell-enriched cell fractions from such animals, spleen cells derived from the same animal were added as described under Material and Methods; cultures to which normal spleen cells were added in the same concentration served as controls. Both sets of cultures were incubated with MTV and per cent inhibition was calculated. The results are shown in Fig. 2. From the results, it is clear that suppressor cells are present early during the growth of the tumour: the first significant inhibition was seen at a tumour weight of 0·5 g. Greatest inhibition is seen at a tumour weight between 0·5 and 1·5 g; also at about 4 g a considerable inhibition occurs.

In animals bearing large tumours, spleen cell cultures containing suppressor cells sometimes gave significantly lower counts than did

Table 2. Proliferation of T-cell enriched cell populations of positive control animals, cultured with adherent cells derived from the same mice or from normal animals

Mouse strain	Cell population	Experiment 1			Exp. 2
		Counts/min ± SE* in the presence of RLV	Counts/min ± SE in the presence of MTV	Per cent stimulation	Per cent stimulation
BALB/cfC3H	T cells and adherent cells	1·991 ± 321	3·047 ± 207	50†	98‡
	T cells and normal adherent cells	2·173 ± 207	3·001 ± 57	46§	74‡
GR	T cells and adherent cells	1·805 ± 134	3·698 ± 325	105‡	145‡
	T cells and normal adherent cells	2·157 ± 288	4·025 ± 178	87‡	136‡

*SE: standard error of triplicate cultures.
†Not significant.
‡$P < 0{\cdot}010$.
§$P < 0{\cdot}025$.

Table 3. Influence of adherent cells from normals or mice bearing tumours on T cell proliferation in the presence of MTV

Mouse strain	Tumour weight (g)	T cells and adherent cells Counts/min ± SE*	T cells and normal adherent cells Counts/min ± SE	Significance	Per cent inhibition
BALB/cfC3H	1·4	1·428 ± 128	2·371 ± 23	$P < 0{\cdot}001$	40
	3·8	1·727 ± 256	3·579 ± 420	$P = 0{\cdot}025$	52
GR	2·8	1·025 ± 76	2·084 ± 234	$0{\cdot}025 < P < 0{\cdot}050$	51
	4·2	1·810 ± 190	3·534 ± 337	$0{\cdot}005 < P < 0{\cdot}010$	49

*SE: standard error of triplicate cultures.

the RLV-control; removing the suppressor cells gave equal values for MTV and RLV cultures; even then, no MTV-specific proliferation was obtained. This was not seen in animals bearing small tumours. This is demonstrated in Table 4. Average per cent stimulation of T-cell-enriched cell fractions of 9 animals with small tumours (average 0·9 ± 0·2 g) was 83·1, and varied from 23 to 154; for 10 animals tested with large tumours (average 3·5 ± 0·2 g) this value was 15·2 with a variation of 0–62%.

Neither antigen nor antibody could be detected on the surface of adherent suppressor cells by membrane fluorescence studies.

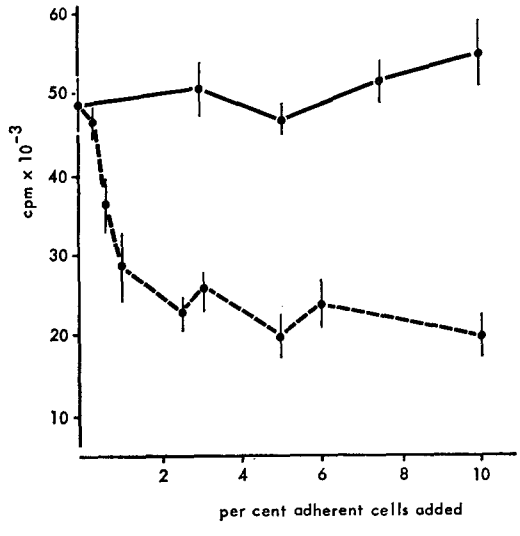

Fig. 1. Inhibition of MTV-specific response of T-cell-enriched cell populations derived from 3 BALB/cfC3H mice bearing very small tumours (average 0·27 g) when varying amounts of adherent suppressor cells derived from 4 BALB/cfC3H mice bearing large tumours (average 3.10 g) are added.

——— = *normal adherent cells added.*
– – – – = *suppressive adherent cells added.*
Vertical bars indicate standard error of triplicate cultures. This figure represents one out of two experiments.

DISCUSSION

From results with the fractions enriched for T-cells it can be concluded that the MTV-specific blast formation observed in splenic cell cultures derived from BALB/cfC3H and GR mice primed with MTV is due primarily to activities of T cells. Blair and Lane [1] claim that a major part of mammary tumour cell killing in the MC test is T-cell dependent. They also report on antibody-dependent null cell activity in this system. Such activity could not be detected in our leukocyte stimulation assays. Fractions enriched for B cells which also would contain null cells did not give any significant proliferation upon incubation with MTV.

The addition of adherent cells derived from positive control animals did not enhance the blastogenesis of T cells as compared to when normal adherent cells were added. It is therefore not likely that macrophages are required

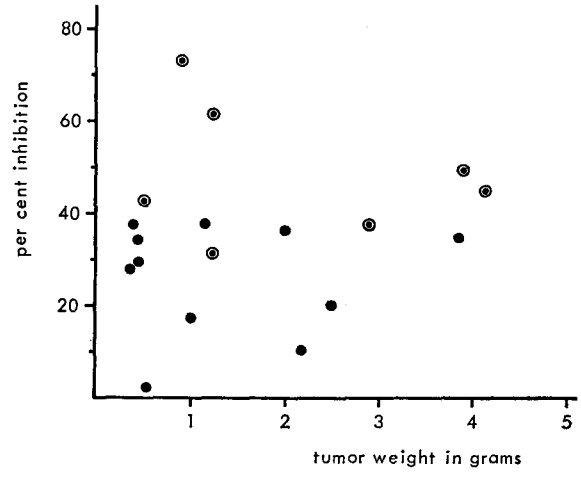

Fig. 2. Percent inhibition of MTV-specific blastforming caused by suppressor cells from individual BALB/cfC3H mice bearing tumours of different sizes.

● : *inhibition is not significant*
⊙ : *inhibition is significant* ($P < 0.025$).

Table 4. Inhibition of MTV-specific and of nonspecific T cell response by suppressor cells present in the spleens of BALB/cfC3H mice bearing spontaneous MTV-induced tumours

Tumour weight	Cell population	Counts/min ± SE* in the presence of RLV	Counts/min ± SE in the presence of MTV	Significance	Per cent stimulation
1·40	Spleen cells	3·464 ± 127	4·272 ± 106	$0.010 < P < 0.005$	24
	T-cell enriched	2·650 ± 664	6·747 ± 380	$0.005 < P < 0.001$	155
2·90	Spleen cells	5·611 ± 342	3·856 ± 98	$0.010 < P < 0.005$	—
	T-cell enriched	2·446 ± 368	2·216 ± 929	N.S.†	0

*SE: standard error of triplicate cultures.
†N.S.: not significant.

in large quantities for induction of specific T cell proliferation. In the previous paper the LS and the LAI tests gave parallelar results in the MTV-system. Holt [8] claims that the production of LAIF, which is responsible for the inhibition of adherence of neighbouring leukocytes, is T-cell-dependent. We have confirmed this for the MTV-system (Creemers, unpublished results). It seems that T cells play a major part in different systems of cellular reactivity to MTV.

In the present study, it was demonstrated that the MTV specific response in tumour bearing animals was depressed by the presence of adherent cells. This suppression appeared not to be a tissue culture artifact, since increasing the cell density by adding an equal number of normal adherent cells had no inhibitory effect. The suppressive effect was already seen when about 0·5% adherent suppressive spleen cells were added to a responding cell population and about 5% was enough to reach the maximal inhibitory effect. These results are in accord with those of Glaser *et al.* [3] in rats bearing progressively growing Gross Leukemia Virus induced tumours. Kirchner *et al.* [4, 5] found suppression of the PHA response of lymphocytes from mice bearing primary MSV induced tumours. Surprisingly, they found an inverse relationship between the development of suppressor cells and virus-specific cytotoxicity. It is therefore possible that no depression will be found in T-cell-dependent cytotoxicity in the MC test with increasing tumour weights. However, Eggers and Wunderlich [9] reported the presence of adherent suppressor cells in the MC test in methylcholanthrene induced tumour bearing animals.

Kirchner *et al.* [4, 5] observed a good correlation between the growth pattern of MSV-induced tumours and suppressor cells were detected before the tumour had reached an appreciable size. In this system, suppressor cells could already be demonstrated at a tumour weight of 0·5 g. The greatest inhibitory effect was seen at a tumour weight between 0·5 and 1·5 g; there was also a considerable effect at 4 g. The decline in spleen cell reactivity when the tumour exceeds one gram in weight can be attributed to increasing suppressor cell activity. At 2 g, when the spleen cell activity in the blastogenesis test is at a minimum (preceding paper), the maximum suppressor cell activity has already disappeared.

Removal of adherent cells did not result in a significant T cell reaction at heavier tumour weights in most cases. Thus, suppressor cells cannot be the only cause of the hyporeactivity observed at a large tumour burden; other factors which make the T cell unresponsive must be involved. Studies on this subject are now in progress in our laboratory.

The mode of action of suppressor cells remains unclear. Membrane fluorescence studies for the detection of antigen or antibody on the surface of the suppressor cells gave negative results. Eggers and Wunderlich [9] found no evidence for the mediation of suppression by soluble factors. In this study it was not investigated whether the suppressor adherent cells are T lymphocytes [10]. Suppressor cells are found only in cases in which animals are overloaded with antigen and the possibility must be considered that suppressor cells are part of a tolerance inducing mechanism that is activated when the immune system is confronted with excess tumour antigen.

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Cryopreserved Autologous Marrow Infusion Following High Dose Cancer Chemotherapy*

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Abstract—*Seventeen patients with advanced malignancies received escalating doses of chemotherapy with adriamycin and cyclophosphamide. Sixty-five per cent of the courses produced circulating granulocyte counts of* 500 *cells/mm*^{3} *or less. Febrile episodes occurred in only* 15% *of courses and were seen only with doses of chemotherapy that produced granulocyte nadirs of less than* 200 *cells/mm*^{3}. *There were no episodes of septicaemia. Ten of the* 17 *patients received an intensive dose followed by reinfusion of cryopreserved, autologous bone marrow. In* 5 *of these patients, recovery to* 500 *granulocytes/mm*^{3} *was more rapid following the intensive, marrow-supported course by comparison with the prior, less intensive, unsupported course. We conclude that higher doses of adriamycin and cyclophosphamide than are conventionally used can be given without serious toxicity. Autologous bone marrow reinfusion may have a role in reducing the period of drug-induced granulocytopenia, but effective storage and recovery of human bone marrow remains a major problem.*

INTRODUCTION

MOST evidence from experimental animal systems indicates that the dose–response curves for antitumour effect and toxicity are steep [1, 2]. For most antitumour agents, myelosuppression is dose limiting. An increase in the severity of granulocytopenia has been shown to predispose to the risk of infection in patients with acute leukaemia [3]. Likewise, in patients with solid tumours, fatal infections are becoming more frequent, possibly related to more intensive and more widespread use of chemotherapy [4].

Reinfusion of autologous bone marrow has been used experimentally in attempts to circumvent myelosuppression, but the efficacy of this approach has not yet been fully established in man [5–8]. In animals, however, effective marrow reconstitution has been achieved [9–11], and has resulted in prolonged survival. Moreover, myelosuppression from sublethal treatment has been effectively shortened by infusion of fresh or cryopreserved isogeneic bone marrow [12].

The chief aim of this study was to investigate the haematological tolerance and safety of high doses of chemotherapy. Cryopreserved autologous bone marrow was employed as a potential means of reducing the duration of drug-induced granulocytopenia, thus permitting the safer administration of high dosage of myelotoxic agents. Each patient served as his own control. This report represents an initial summary of our experience with this approach and emphasizes the haematological response and the problems of marrow storage and reinfusion. The 10 patients who have

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completed the programme are also evaluated for tumour response.

MATERIAL AND METHODS

Patient population and chemotherapy design

Seventeen patients with tissue-proven metastatic carcinoma were entered into this study. Eleven patients had stage III/IV ovarian cancer, untreated except for previous surgery. One patient had stage III ovarian cancer previously treated with surgery and chemotherapy. One patient had stage III teratocarcinoma of the testis previously treated by surgery. One patient had primary peritoneal mesothelioma. Three patients had carcinoma of the breast. Of these, one had been treated with chest wall irradiation following simple mastectomy. This patient underwent adrenalectomy and oophorectomy prior to entry into this study, and was given replacement steroid therapy throughout the period of treatment. Two further patients were treated with radical mastectomy and chest wall irradiation. In one of these patients, irradiation was given concurrently with low-dose chemotherapy prior to treatment with high-dose chemotherapy on this programme. In the other patient, irradiation was completed eight months before entry into the study.

All patients were treated with increasing doses of adriamycin (ADR) and cyclophosphamide (CY) according to the scheme presented in Fig. 1. Initial doses were ADR 45 mg/m^2 and CY 500 mg/m^2. All drugs were given intravenously over two consecutive days (ADR day 1, CY day 2), and dosage was rapidly escalated at three-weekly intervals.

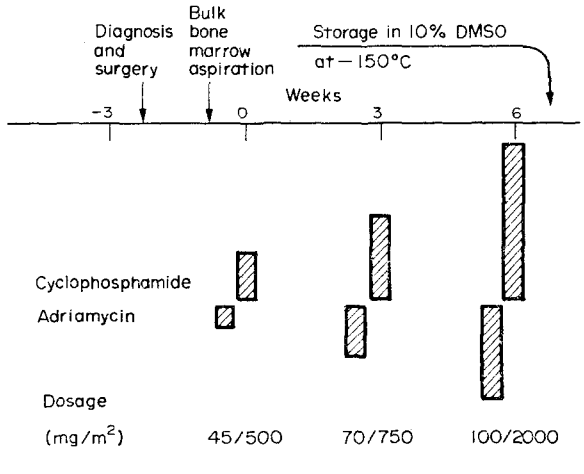

Fig. 1. Schematic presentation of intensive chemotherapy programme with autologous marrow aspiration, storage and reinfusion. Chemotherapy was rapidly escalated and marrow reinfusion took place 72 hours after the intensive course.

Haematological response was closely monitored and all patients had complete blood counts including differential and platelet counts at least 3 times weekly and more frequently during periods of critical myelosuppression. All patients received at least one course of therapy consisting of ADR 70 mg/m^2 plus CY 750 mg/m^2. Ten patients received a more intensive course usually consisting of ADR 100 mg/m^2 and CY 2000 mg/m^2. The additional seven patients are included in the evaluation of marrow procurement and haematological tolerance to the chemotherapy. A total of 99 courses of ADR/CY was given. No patient received more than 450 mg/m^2 of ADR as a total dose.

Marrow aspiration and cryopreservation

Prior to chemotherapy, bulk marrow aspiration was carried out under general anaesthesia by a modification of the method of Thomas and Storb [13]. Briefly, marrow was obtained from the posterior superior iliac spines and posterior iliac crests using heparin (Weddel Pharmaceuticals, London) 50 units/cc in Hanks' balanced salt solution as an anticoagulant. After filtration to remove marrow spicules, mononuclear cells were concentrated by centrifugation. The net number of marrow cells was calculated by subtracting peripheral blood leukocytes from the total nucleated cell count of the bulk marrow aspirate. Dimethylsulfoxide (DMSO) was added to a final concentration of 10%, and the marrow was cooled in Hemoflex bags (Union Carbide, Chicago, Illinois) at 1°C/min to −40°C and then rapidly to −100°C in an automated liquid nitrogen freezer (Planer Instruments, Middlesex). The sample was maintained in the vapour phase of liquid nitrogen (−150°C) for 2 to 4 months. The viability of myelocytic progenitor cells was assessed both in fresh specimens and in aliquots of cryopreserved specimens by the *in vitro* colony assay (CFU-C) using the method of Iscove *et al.* [14].

Infusion of autologous bone marrow

In all 10 patients receiving the intensive ADR/CY course the autologous marrow was reinfused 72 hr after the administration of ADR. Marrow was rapidly thawed in a 40°C water bath, diluted with plasma/saline, centrifuged and resuspended for infusion as described elsewhere [15]. An aliquot of the infused marrow was used to determine the total number of cells, viability by trypan blue exclusion and CFU-C content. In all cases, the level of ADR equivalents at the time of marrow in-

fusion was determined using the radioimmunoassay described by Van Vunakis *et al.* [16]; more complete ADR clearance studies were carried out in five patients.

The ability of autologous bone marrow to alter the period of granulocytopenia was evaluated by comparing total granulocyte counts during the intensive marrow-supported course (transplant course) with granulocyte counts during the prior, less intensive and unsupported course of chemotherapy (pre-transplant course). Specifically, the number of days spent with granulocyte counts less than 500/mm^3 for these 2 courses were compared in each patient. The granulocyte and platelet nadirs for the courses were also compared. Effectiveness of marrow infusion was assessed by determining the number of days from the start of therapy to the day at which recovery to 500 granulocytes/mm^3 took place.

Evaluation of tumour response

Evaluation of response in this report is limited to those patients who underwent reinfusion of autologous marrow. Complete response (CR) refers to the disappearance of all objective evidence of disease for a period of at least two months. Partial response (PR) refers to reduction of measurable tumour by greater than 50%. Patients who responded less than 50% or who had no change in their tumour status were designated as having stable disease (SD). All other patients were considered to have tumour progression (TP). Subjective improvement was not a criterion for response. For patients with ovarian cancer, all responses were verified by laparoscopy and laparoscopic biopsy of areas of disease known to be present after primary surgery.

RESULTS

Bone marrow aspiration and processing

In the 17 patients from whom marrow was aspirated, the median yield of bone marrow cells was $1{\cdot}9 \times 10^{10}$ (range $0{\cdot}9$–$2{\cdot}9 \times 10^{10}$) (Table 1). The median net number of nucleated bone marrow cells after correction for peripheral blood contamination was $1{\cdot}0 \times 10^{10}$ (range 0–$1{\cdot}8 \times 10^{10}$). There was no clear relationship between the volume of bone marrow aspirated and the net cell harvest. After processing, the median number of cells preserved was $1{\cdot}3 \times 10^{10}$ (range $0{\cdot}6$–$2{\cdot}8 \times 10^{10}$). This figure includes peripheral blood mononuclear cells and is therefore higher than the figure for the net yield of bone marrow harvested. Marrow aspiration resulted in a fall in hematocrit from $36{\cdot}3\% \pm 1{\cdot}5$ to $29{\cdot}8\% \pm 1{\cdot}6$ in 8 patients who were not transfused; the other 9 patients received red cell support immediately following the aspiration.

Table 1. *Bone marrow aspiration and storage*

Pt.	Net vol. aspirate (ml)	Total cells aspirated ($\times 10^{10}$)	Net marrow cells ($\times 10^{10}$)	Total cells frozen ($\times 10^{10}$)
1	935	1·9	1·0	1·2
2	1000	2·9	1·8	2·8
3	824	1·7	0·9	1·3
4	1121	1·9	1·1	1·8
5	960	1·9	1·0	2·6
6	926	1·5	0·8	1·3
7	1021	1·6	1·1	1·2
8	835	1·8	0·9	1·3
9	321	0·9	0·7	0·6
10	1401	2·7	1·6	1·9
11	637	1·8	1·1	1·3
12	764	1·6	1·0	1·1
13	1032	2·2	1·3	1·1
14	1137	2·8	*	1·9
15	393	1·6	1·3	1·6
16	711	1·9	1·5	1·3
17	1152	1·9	1·1	1·2
Median	935	1·9	1·0	1·3

*Leukocyte counts on bone marrow aspirate and peripheral blood were 27,000 and 28,000 respectively.

This table shows data on aspiration, processing and storage of bone marrow. "Net marrow cells" refers to gross cell yield minus cells derived from the peripheral blood (obtained by subtracting peripheral white blood count multiplied by volume of aspirate, from total cells aspirated). "Total cells frozen": usually less than gross yield because of losses in processing. Greater than "net marrow cells", since peripheral blood mononuclear cells are included.

Assays for CFU-C content were performed on 4 ml aspirates obtained from each patient prior to bulk bone marrow harvest. In 14 patients, the median CFU-C concentration was 53 (range 7–117)/10^5 sedimented cells.

Myelosuppression and fever

The mean granulocyte nadir following doses of ADR 45/m^2 and CY 500 mg/m^2 was 695 ± 75/mm^3 (Table 3), and the mean platelet nadir was $198{,}000 \pm 12{,}000$/mm^3. At the higher dose of ADR 70 mg/m^2 and CY 750 mg/m^2, the mean granulocyte and platelet nadirs were 266 ± 39/mm^3 and $147{,}000 \pm 10{,}000$/mm^3 respectively. In the 3 patients who had received previous irradiation, both the granulocyte and platelet nadirs were lower; at the dose of ADR 45 mg/m^2 and CY 500 mg/m^2, the difference for both granulocytes and platelets

was statistically highly significant ($P < 0{\cdot}001$, Mann–Whitney U Test).

A characteristic curve of granulocyte depression following chemotherapy was derived by superimposing the granulocyte counts from all courses at each dose level (Fig. 2). The curves shown represent the median of these points. Similar curves were drawn for the platelet counts. At the dose of ADR 70 mg/m^2 and CY 750 mg/m^2, the rise in total leukocyte count preceded granulocyte recovery. This was due to an absolute monocytosis commencing on day 12 (Fig. 3).

Table 2. Bone marrow reinfusion

Pt.	Total cells reinfused ($\times 10^{10}$)	Total cells per kg ($\times 10^8$)	Viable cells per kg ($\times 10^8$)	Total CFU-C reinfused ($\times 10^6$)
1	1·0	1·7	0·9	0·4
2	1·6	2·8	1·4	1·1
3	1·3	2·2	1·1	3·5
4	1·1	1·5	0·8	0·4
5	0·7	1·6	0·8	0
6	0·8	1·1	0·2	0
7	0·8	1·2	0·8	0·3
8	0·6	1·6	0·5	0·4
9	0·6	1·0	0·7	2·8
10	1·2	2·9	1·9	2·2
Median	0·8	1·6	0·8	0·4

This table shows data on reinfusion of cryopreserved bone marrow. "Total cells reinfused": usually less than "total cells frozen" because of losses during freezing. "Viable cells" refers to Trypan blue exclusion. Total CFU-C reinfused was calculated by total volume of infusion multiplied by CFU-C content of marrow.

In the 99 courses of chemotherapy, granulocytopenia to less than 500 cells/mm^3 occurred in 64 courses (65%), and in 29 of the 64 courses, the granulocyte nadir was less than 200 cells/mm^3. Of the latter 29 courses, 14 were complicated by fever (48%) (Table 4). Only one additional febrile episode occurred during this study; this was the result of a wound infection following paracentesis in a patient whose granulocyte count was greater than 1000 cells/mm^3.

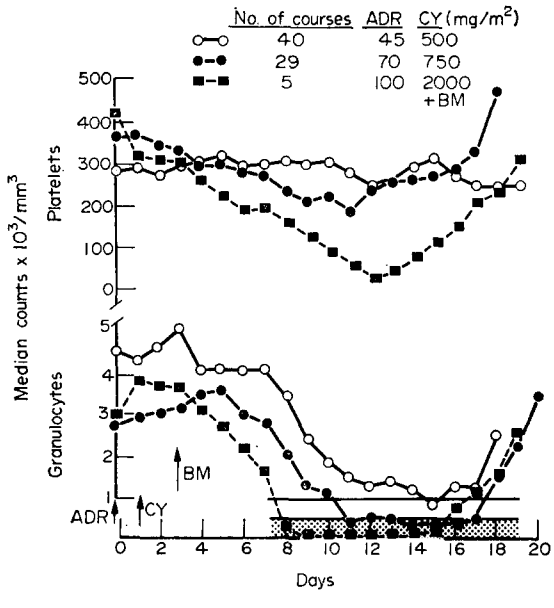

Fig. 2. Myelosuppression following increaing doses of ADR/CY chemotherapy. Curves were obtained by superimposing the counts from all courses for each dose and taking the median. Bone marrow was reinfused 72 hours after the intensive course. Horizontal lines show duration of granulocytopenia at 1000 cells/mm^3 and 500 cells/mm^3 (hatched). BM: bone marrow reinfusion.

Table 3. Haematological tolerance to ADR/CY chemotherapy

Dose (mg/m^2)	All courses	Prior X-ray	No prior X-ray
ADR 45/CY 500			
Number of courses	40	11	29
Gran nadir (per mm^3)	695 ± 75	399 ± 53	797 ± 96
Platelet nadir ($\times 10^3$/mm^3)	198 ± 12	146 ± 16	221 ± 15
ADR 70/CY 750			
Number of courses	29	4	25
Gran nadir (per mm^3)	266 ± 39	215 ± 146	270 ± 37
Platelet nadir ($\times 10^3$/mm^3)	147 ± 10	97 ± 28	160 ± 10

All values represent mean ± 1 S.E.M.

This table shows absolute granulocyte and platelet nadirs for two doses of chemotherapy. Sixty-nine courses of chemotherapy are analysed: 40 courses of ADR 45 mg/m^2 and CY 500 mg/m^2, and 29 courses of ADR 70 mg/m^2 and CY 750 mg/m^2. Previously irradiated patients experienced lower nadirs (both granulocyte and platelet counts) than patients not previously irradiated. For the lower dosage, this difference was statistically highly significant ($P < 0{\cdot}001$, Mann–Whitney U test).

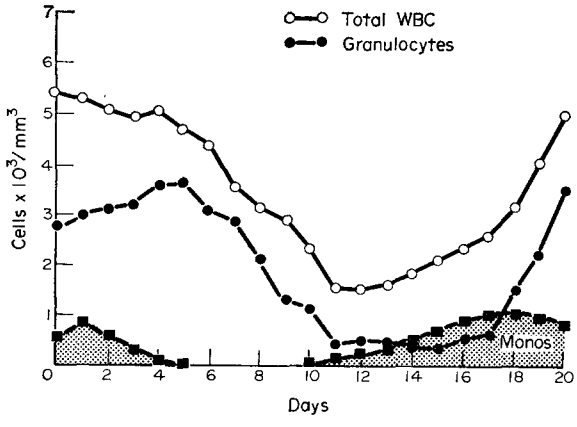

Fig. 3. Total leukocyte, granulocyte, and monocyte counts following ADR 70 mg/m² and CY 750 mg/m². Curves were obtained by superimposing the counts from all courses and taking the median. Monos: absolute circulating monocyte count.

Table 4. Fever and granulocytopenia

Depth

Granulocyte nadir (cells/mm³)	Total courses	Febrile courses	
500–400	4	0	0%
399–300	17	0	
299–200	14	0	
199–100	7	1	48%
<100	22	13	

Duration

Duration of grans <200 (days)	Total courses	Febrile courses	
1–2	7	0	(0%)
3–6	19	11	(58%)
7–8	3	3	(100%)

This table shows the relationship between granulocytopenia and the likelihood of fever during that course of chemotherapy. No fevers were observed in the 35 courses of chemotherapy which produced granulocyte nadirs between 200 cells/mm³ and 500 cells/mm³. When this nadir was exceeded, the risk of fever was 48% (14 febrile courses out of 29 courses). Fever also correlated with duration of granulocytopenia. An increased duration of granulocytopenia carried a steeply increasing risk of fever.

The number of febrile episodes was also related to the duration of granulocytopenia. Fever occurred in 0/7 (0%) courses in which granulocytopenia (less than 200 cells/mm³) lasted less than 3 days, in 11/19 (58%) courses in which granulocytopenia lasted 3–6 days, and 3/3 (100%) courses in which granulocytopenia lasted 7–8 days. In only 2 courses was there bacteriological documentation of infection despite vigorous attempts at culturing blood, urine, sputum, nose and throat. One patient had a staphylococcal cellulitis and one had a coliform urinary infection. There were no signs of septicaemia in any of the patients, and all recovered within seven days after the onset of fever.

Non-haematological toxicity

Alopecia was universal and was usually complete after three courses of ADR/CY therapy. Mild mucositis, consisting of buccal ulceration and soreness, occurred in 0/40 (0%) courses of ADR 45 mg/m² and CY 500 mg/m²; in 2/29 (7%) courses of ADR 70 mg/m² and CY 750 mg/m²; and in 2/8 (25%) courses of ADR 100 mg/m² and CY 1000–2000 mg/m². Mild, transient, haemorrhagic cystitis occurred during two courses of ADR 70 mg/m² and CY 750 mg/m² in one patient. Nausea and vomiting occurred in almost all patients following CY administration. It was usually transient (mean duration of vomiting 8 hr; mean duration of nausea 24 hr) and well controlled with oral, intramuscular or rectal prochlorperazine and/or pentobarbitone. Since heavy sedation was given to all patients receiving high dose ADR/CY therapy, it was not possible to correlate dosage with degree of nausea and vomiting. In 12/99 (12%) courses, there was significant residual nausea and/or vomiting which lasted beyond 24 hr. All patients had frequent electrocardiographic examinations prior to and during therapy. No patient developed signs or symptoms of cardiac failure at any time. In one patient, proteinuria had been noted prior to therapy. In this patient, transient nephrotic syndrome developed during therapy. Renal function remained normal and all signs of nephrotic syndrome (including proteinuria) resolved within the two months following intensive therapy.

Bone marrow infusion and haematological recovery

In the 10 patients receiving bone marrow transfusion, the median number of mononuclear cells thawed and reinfused was $0{\cdot}8 \times 10^{10}$ (range $0{\cdot}6$–$1{\cdot}6 \times 10^{10}$) (Table 2). Median Trypan blue viability was 50% (range 18–70%). An aliquot of the final marrow suspension yielded a median CFU-C concentration of $6{\cdot}0/1 \times 10^5$ cells plated (range 0–46). The median number of CFU-Cs infused was 4×10^5 (range 0–25). This was derived from the product if the total number of cells and the CFU-C content of the infused marrow.

In all patients the marrow reinfusion was

accompanied by the typical pungent odour associated with DMSO infusion. The estimated total dose of DMSO was 1–5 g per patient. Five patients experienced no difficulty with the marrow infusion. Two patients had paroxysmal cough for approximately one hour, and chest X-rays were negative in both. One patient had chills and weakness for 2 hr, and three patients complained of local irritation and burning at the injection site. Five patients were reinfused with bone marrow as outpatients and all of the patients were hospitalized when the granulocyte count reached 500 cells/mm^3 or less.

Following intensive chemotherapy with ADR 100 mg/m^2 and CY 2000 mg/m^2, granulocyte counts dropped more rapidly by comparison with previous courses. The median granulocyte count by day 8 was 300 cells/mm^3, in contrast to a median granulocyte count of 2000 cells/mm^3 in patients given ADR 70 mg/m^2 plus CY 750 mg/m^2. In 5 of 10 patients, recovery from the start of therapy to 500 granulocytes/mm^3 was more rapid in the transplant course than the pre-transplant course (Table 5). In 3 cases, the rate of recovery was the same, and in 2 cases, it was slower. The absolute granulocyte nadir was 245 ± 89/mm^3 in the pre-transplant courses and 42 ± 23/mm^3 in the transplant courses. Thus, recovery from the transplant courses was at least as rapid as from the pre-transplant courses despite a more profound granulocyte nadir. However, the number of days spent with a circulating granulocyte count of less than 500 cells/mm^3 was greater (6·7 days) for the transplant course than for the pre-transplant course (4·5 days) (Table 5). This was entirely due to the earlier fall in granulocyte counts produced by the more intensive ADR/CY therapy (Fig. 2). In one patient, recovery during the transplant course was particularly rapid (Fig. 4).

At the time of marrow transfusion, the median plasma ADR level was 10 nM, with a range of 6–15 nM (Fig. 5). There was little interpatient variability in ADR clearance. In the patient who demonstrated the most rapid recovery of circulating granulocytes, the level of ADR at 72 hr was 15 nM. This value was similar to the levels observed in the other patients.

Tumour response

Nine of the 10 patients who received intensive therapy with marrow reinfusion were evaluable for antitumour response. Six of these patients had stage III/IV ovarian cancer, one had peritoneal mesothelioma, one had teratocarcinoma and one had carcinoma of

Table 5. *Time to recovery from myelosuppression following intensive chemotherapy with marrow support*

Pt	Dose ADR/CY (mg/m^2)		Granulocyte nadir (grans/mm^3)‡		Days to recovery of 500 grans/mm^3		Days at < 500 grans/mm^3	
	Pre*	Tx†	Pre	Tx	Pre	Tx	Pre	Tx
1	70/750	100/2000	750	0	19	16	4	7
2	45/500	70/750	351	115	19	17	5	7
3	70/750	100/1000	242	16	18	13	6	3
4	70/750	100/1000	232	0	17	16	3	7
5	70/750	100/2000	77	18	17	17	6	8
6	45/750	70/750	77	222	17	17	5	5
7	45/750	100/2000	59	5	16	15	5	6
8	45/1000	60/2000	67	46	16	16	3	9
9	100/1000	100/2000	54	0	16	17	7	8
10	45/500	100/2000	980	0	—	15	0	7
			245 ± 89	42 ± 22	17 ± 0·4	16 ± 0·4	4·5 ± 0·6	6·7 ± 0·5

*Pre-transplant course.
†Transplant course.
‡Grans: granulocytes.
All values represent mean ± 1 S.E.M.

This table shows data on intensive chemotherapy, bone marrow reinfusion and haematological recovery (10 patients). Granulocyte nadir was more profound after the intensive chemotherapy by comparison with the previous course, but the number of days from start of therapy to recovery (500 granulocytes/mm^3) was fewer following intensive chemotherapy/marrow reinfusion, in 5 patients. However, absolute number of days spent with a circulating granulocyte count < 500 cells/mm^3 was usually greater in the intensive course, since intensive chemotherapy produced a more rapid fall in the circulating granulocyte count (see Fig. 2).

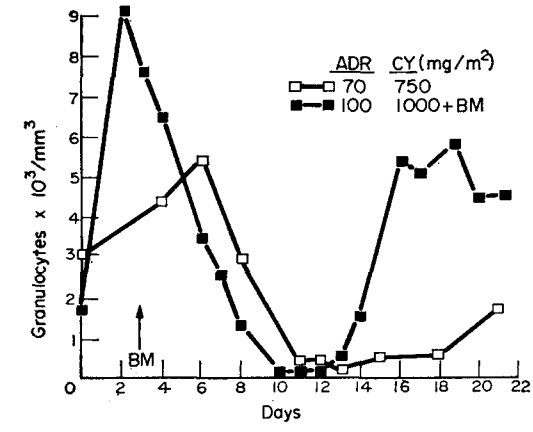

Fig. 4. Granulocytopenia in patient No. 3 following intensive chemotherapy with and without marrow support. BM: bone marrow reinfusion. Duration of granulocytopenia was reduced during the marrow-supported course despite increase in chemotherapy dosage.

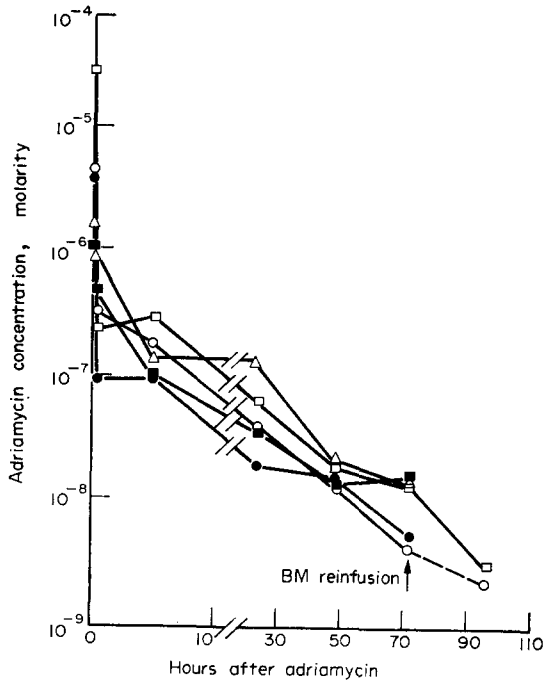

Fig. 5. Plasma clearance of ADR in patients undergoing reinfusion of autologous bone marrow. Four patients received ADR 100 mg/m² at time zero: one patient (●——●) received ADR 60 mg/m² at time zero. (Dotted line represents value below limit of assay.)

the breast. Objective responses were seen in 6 patients. Four of these responses (1 complete) were seen in the 6 patients with stage III/IV ovarian cancer. A PR was seen in one of the patients with breast cancer and the patient with teratocarcinoma, and no response was seen in the patient with peritoneal mesothelioma. Further data on ADR/CY in patients with advanced ovarian cancer has been presented by us elsewhere [17].

DISCUSSION

Attempts to demonstrate the value of autologous marrow following chemotherapy in man have been frustrated by the relatively short duration of granulocytopenia even with very intensive doses of drugs [18]. Buckner *et al.* gave high doses of CY (120 mg/kg) followed by autologous marrow to patients with a variety of disseminated malignancies [5]. No firm conclusions regarding the value of autologous marrow could be drawn although there was a slight trend towards earlier recovery from leukopenia in the reinfused patients. Previous work had shown that in primates, spontaneous recovery from CY occurred so rapidly that the additional benefit of infused marrow could not be documented [19]. We anticipated that improved haematological recovery following marrow infusion would be easier to demonstrate in patients given combination ADR/CY therapy than in patients given CY alone since the granulocyte nadir following ADR characteristically occurs later [20].

In only one patient was there clear-cut reduction in the duration of granulocytopenia following bone marrow reinfusion. We assumed that this was due to early reconstitution of bone marrow from the reinfused stem cells. In four of the other patients, recovery to 500 granulocytes/mm³ from the marrow-supported course was more rapid that it was after the prior, less intensive course. However, the overall duration of granulocytopenia was greater during the intensive course, and this was entirely due to the more rapid fall in granulocyte count seen with the larger dose of chemotherapy. Thus is would appear that the infused bone marrow contributed to earlier reconstitution but that no clinical benefit was derived because the onset of granulocytopenia was so rapid following intensive chemotherapy.

The viability of the transfused marrow, as measured by the CFU-C assay, was poor in most patients (Table 2). Although long-term preservation of murine, canine and primate bone marrow has been repeatedly accomplished [21–23], there is little evidence that large volumes of marrow can be successfully cryopreserved in man. Gray and Robinson demonstrated that storage up to 14 days did not result in significant loss of CFU-C [24]. Ragab *et al.* noted a substantial decrease in CFU-C, however, after cryopreservation of leukaemic marrow for periods of up to 16 weeks [25]. Schaefer and Dicke demonstrated

that the technique of dilution after thawing may be critical for optimal CFU-C recovery [26], and several methods are currently being studied. In our series, cell recovery was 62% and trypan blue viability was 50%. The CFU-C recovery was variable despite optimal rate of cooling, temperature of storage and choice of cryopreservative.

Attempts to establish the value of intensive chemotherapy have been hindered by morbidity from myelosuppression. Buckner *et al.* demonstrated that high doses of CY alone were capable of inducing responses in sensitive tumours, although it was not demonstrated that the response rates were unequivocally higher than those seen with more conventional dosage [5, 27, 28]. The present study has shown that it is possible to deliver intensive combination therapy without serious toxicity to the patient. Non-myelosuppressive toxicity was acceptable, and rarely precluded dose escalation. Although myelosuppression was frequently severe (65% of courses producing granulocyte counts less than 500/mm^3) there were no episodes of life-threatening infection. There was a clear relationship between fever and granulocytopenia. No fevers were seen in the 30 courses of therapy which produced granulocyte counts between 200 and 500 cells/mm^3. The risk was considerably increased only if this nadir was exceeded. Patients experiencing three or more days with circulating granulocyte counts less than 200 cells/mm^3 were more likely to become febrile (i.e., presumably infected) with increasing periods spent at this level. The study by Bodey *et al.* in patients with acute leukaemia clearly demonstrated a close relationship between granulocytopenia and probability of infection at all levels up to 1500 granulocytes/mm^3 [3]. Clearly, patients with solid tumours can tolerate a far greater degree of granulocytopenia than patients with acute leukaemia; this difference probably reflects the superior marrow reserve of patients with malignancy not involving the marrow, by comparison to those with a haematological malignancy.

This study therefore supports the concept that patients with solid tumours can tolerate more intensive chemotherapy than is usually employed. In addition, reinfusion of autologous marrow may play a part in hastening marrow recovery from such therapy, although satisfactory storage of human bone marrow with reliable maintenance of viability has not yet been achieved. Future investigation and use of autologous bone marrow transplantation as support for intensive chemotherapy will depend largely on improvements in storage and recovery techniques. It is not yet possible to assess duration of response to high-dose chemotherapy in this series, but the initial findings are encouraging.

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The Effect of Methotrexate on the Uptake of *De Novo* and *Salvage* Precursors into the DNA of Rat Tumours and Normal Tissues

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Abstract—*The effects of two single doses of methotrexate (MTX) upon the incorporation of labelled* de novo *and* salvage *precursors into the DNA of tumour (BICR A*15*-kidney carcinoma), bone marrow, intestinal mucosa and spleen have been monitored in inbred August female rats. Fractional incorporation, used as an index of therapeutic response, has proved effective in estimating levels of tissue DNA synthesis. MTX has been shown to exert its cytotoxic effect in rats by blocking the* de novo *synthesis of thymidylate. The intestinal mucosa was shown to be extremely sensitive to MTX treatment. A dose of* 14 *mg/kg destroyed large areas of mucosa and resulted in the death of the animal after* 4 *days. The tumour appeared to overcome the MTX induced reduction in* de novo *thymidylate synthesis by utilising a* salvage *supply of thymidine thus by-passing a potentially lethal block through an alternative metabolic pathway. This difference between the response of the tumour and of a critical normal tissue, the intestinal mucosa, would severely limit the effectiveness of MTX for tumour therapy.*

INTRODUCTION

THE PRESENCE of alternative metabolic pathways in both normal and tumour cells is a factor which can lead to complications when scheduling cytotoxic drug treatment. The relevance of such anabolic alternatives has been realized for many years [1]. However, a further complication is that cells are rarely in a biochemical "steady state" condition. A given cell type is usually "totipotent" with respect to the various pathways to a given product. Sneider and Potter [2] have shown that cells may turn on to different metabolic pathways during various stages of differentiation. Since tumour cells may be regarded, in some cases, as cells frozen at various stages of differentiation [3, 4], information concerning essential metabolic pathways used by tumour and normal cells, proves important in consideration of possible vital tissue sparing drug regimens.

Methotrexate (MTX) is clinically a widely used antimetabolite [5], acting through competitive inhibition of the enzyme dihydrofolate reductase (EC 1.5.1.3.). Current concepts of the specific cytotoxic action of MTX are varied. Inhibition of *de novo* thymidylate production [6, 7] and inhibition of *de novo* purine production [8] have been cited. In addition, prevention of amino acid and RNA synthesis [9] or a combination of all three effects [10] have been considered.

The object of this present study was to investigate the cytotoxic action of MTX by following its effect upon the uptake of labelled precursors into DNA. The precursors used and the biochemical pathways in which they participate are shown in Fig. 1, this shows that thymidylate may be synthesised either by *de novo* or *salvage* procedures. The possibility of a *salvage* by-pass of a MTX-induced block in the *de novo* synthesis of thymidylate was considered by monitoring the incorporation of thymidine-3H (TdR) into tissue DNA. Deoxyuridine-3H (UdR) and sodium formate-14C were used as *de novo* precursors. The pattern of response of these three precursors, following drug treatment, has been shown to correlate with

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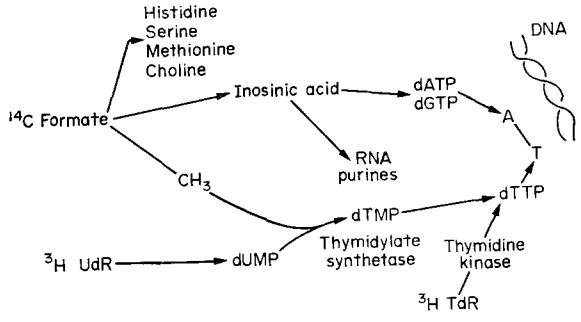

Fig. 1. *Metabolic fate of labelled precursors: simplified biochemical pathways.*

the therapeutic response observed in various tumours and normal tissues [11, 12].

Chabner and Young [13] have demonstrated a MTX induced block in deoxyuridylate to thymidylate by expressing the uptake of UdR-3H into DNA as DPM/μg DNA. Myers *et al.* [14] have shown that the incorporation of UdR into DNA of normal and neoplastic tissues after 5FU is dependent not only upon the rate of conversion of dUMP to dTMP but also on changes in the endogenous dUMP pool sizes. These authors used correction factors which, by correcting to time zero, compensated for alterations in dUMP pool sizes. The method used in this study utilises the systemic distribution of labelled precursors over a one hour pulse label and expresses DNA synthesis as the percent of the total label reaching the tissue which is incorporated into the DNA, i.e.,

$$\text{Fractional incorporation (F.I.)} = \frac{\text{DPM DNA}}{\text{Total tissue DPM}}$$

Thus, whilst this measure takes no account of the chemical form of the label in the acid soluble fraction, it will compensate for large variations in endogenous radioactivity. F.I. also has the advantage of being independent of drug-induced fluctuations in total tissue DNA content; a fact of not inconsiderable importance when dealing with solid tumours [15].

For these reasons F.I. has been used to study the response of both *de novo* and *salvage* DNA syntheses to single dose MTX treatment.

MATERIAL AND METHODS

The animals used in these studies were 5- to 6-month-old inbred August female rats bearing subcutaneous (s.c.) A15 carcinomata. Methotrexate (Lederle Labs) was administered intraperitoneally (i.p.) to lightly anaesthetised rats, in single doses. Thymidine-3H (TdR) 24 Ci/mmol; deoxyuridine-3H (UdR) 21 Ci/mmol and sodium formate-14C (formate) 50 mCi/mmol were also administered i.p. 1 hr prior to death (Radiochemical Centre, Amersham).

Animals were killed by cervical dislocation, tissues removed and stored at −70°C until used. Excised tumours were stripped of encapsulating tissue. All adipose tissue was removed from the spleens. The marrow from both tibias was blown into cold 0·2N perchloric acid. The mucosa was stripped from a 10 to 15 cm segment of jejunum and transferred into 0·2N perchloric acid. An additional 1 cm length of jejunum was fixed in formol saline, sectioned and stained with haematoxylin and eosin.

Nucleic acid extractions were carried out using a modification of the Schmidt–Thannhauser technique [16]. DNA [17] and protein [18] were estimated colourimetrically.

Radioactivity was measured on an ABAC SL 40 β scintillation counter using a scintillant consisting of 7 vol of butyl—PBD (0·6% wt/vol) in toluene plus 3 vol of tergitol TP9 (Union Carbide Ltd.). For dual isotope studies, appropriate corrections were made for quenching a channel crossover. Cross contamination of the DNA fraction with protein or RNA [19] was checked colourimetrically and compensated mathematically.

In order to distinguish labelled thymine, adenine and guanine after formate-14C administration, DNA bases were hydrolysed with 98% formic acid at 175°C for 30 min. The resultant free bases were adsorbed onto acid washed, neutralised charcoal [20] and eluted overnight at 37°C with an ethanol/ammonia/water solution (2:1:2) [21]. This solution was concentrated by evaporation in a stream of warm air. Base separation was achieved by one dimensional PEI cellulose thin layer chromatography with isopropanol/conc. HCl/H_2O (170/41/39 v/v) [22]. Rf values were similar to those obtained with either paper or cellulose t.l.c. i.e., thymine 0·82; cytosine 0·49; adenine 0·38; guanine 0·26.

Areas of cellulose absorbing u.v. light at the Rf values stated were scraped into glass vials and the radioactivity was eluted with 1 ml of 1 N perchloric acid for 2 hr at room temperature. After the addition of 10 ml scintillant, samples were shaken and counted. Counting efficiency was not impaired by the presence of cellulose particles and 95–100% recovery of ^{14}C was achieved. The TLC sheets used were Schleicher and Schull TLC Ready Plastic Sheets F1440 PEI/LS 254 PEI cellulose 20 × 20 cm.

Tumour volumes were calculated from the formula $V = \pi d^3/6$ where d is the mean caliper diameter of the subcutaneous tumour. Drugs were administered to groups of rats when the tumour volumes were about 1 cm^3. Pair feeding of controls involved giving animals in the control groups the same amount of food as was consumed by the MTX treated groups during the previous 24 hr. Both treated and control animals were allowed water *ad libitum.*

Fractional Incorporation values are calculated from the dis/min of each fraction, i.e.,

$$\frac{\text{DNA dmp}}{(\text{DNA} + \text{Acid soluble} + \text{RNA} + \text{Protein dpm})}$$

This measure has been shown to be similar and in some cases superior to [23] specific activity (dis/min per unit DNA) as a method of assessing DNA synthesis.

RESULTS AND DISCUSSION

The differing sizes of the precursor pools in specific individual tissues limit the inter-tissue comparison of either F.I. or specific activity values. This is especially true for comparisons of the three precursors used in this study. Whilst F.I. values are expressed as ratios, and therefore have inherent correction of such pool differences, the chemical nature of the label in the acid soluble fraction is not known. Moreover, with ^{14}C-formate, not only is the label incorporated into DNA-thymine, but also into the purines of DNA and RNA and into protein amino acids. Such diversification of biochemical endpoints means that not all of the ^{14}C is channeled into a single precursor pool. In addition, the balance of these pools within the acid soluble cell fraction may be altered following drug treatment. Table 1 shows the uptake of ^{14}C-formate into the RNA and protein fractions of the same tissue samples used for DNA estimates. MTX reduced protein synthesis only in the gut, for a period of 24–72 hr after treatment. F.I. values which are significantly higher than the control level may be the result of a redirection of ^{14}C from MTX-inhibited DNA synthesis to protein and RNA precursor synthesis. Spleen DNA synthesis was so high at 264 hr post MTX therapy that the supply of ^{14}C to RNA and protein synthesis was restricted. Thus, the values at this point were low, not because of reduced rates of RNA and protein synthesis, but because of a competitive use of label for DNA synthesis. This would suggest that, in an actively dividing tissue, ^{14}C formate is used preferentially for synthesis of DNA precursors.

Control values of F.I. and specific activity for four tissues are shown in Table 2. The specific activity values for the gut were higher than F.I. values of the same samples for all three precursors. In all cases specific activity is dependent upon precursor supply.

MTX has been shown to exert its cytotoxic effect in Ehrlich ascites carcinoma cells [24],

Table 2. *Control values for fractional incorporation and specific activity*

	Spleen	Tumour	Gut	Bone marrow
TdR	82(60)	57(40)	62(190)	91(73)
UdR	37(31)	6(5)	27(40)	57(48)
Formate	41(56)	10(19)	10(28)	48(47)

Figures in parenthesis are specific activities expressed as dis/min per μg DNA.

Fractional incorporation values are as described in text.

Each value is the mean of at least 6 experiments. Standard errors < 10%.

Table 1. *The uptake of ^{14}C-formate into RNA purines and protein amino acids after* 5 *mg/kg MTX*

	RNA				Protein			
	Spleen	Tumour	Gut	Bone marrow	Spleen	Tumour	Gut	Bone marrow
Control	100	100	100	100	100	100	100	100
12	108	93	95	144*	171*	144*	108	151*
24	135*	93	107	167*	136*	144*	87†	121*
48	124*	85	108	145*	158*	111	55†	143*
72	136*	81	106	176*	154*	118	35†	141*
124	127*	106	95	111	152*	91	106	156*
264	65†	108	96	93	77†	96	106	108

Results are expressed as percent of control fractional incorporation. Each is the mean of at least 3 experiments.

*Value statistically significantly high.

†Value statistically significantly low. ($P > 0{\cdot}05$)

spleen tissue [25] and bone marrow [26] by blocking the folate cofactor-mediated methylation of deoxyuridylic acid to thymidylate. In the spleens of mice with advanced L1210 leukaemia, thymine synthesis could proceed even with an impaired folate reductase system [27] and in other studies prevention of *de novo* purine synthesis has been cited as the cytotoxic effect of MTX [28].

Three factors suggested that, in August rat tissues, the cytotoxicity of MTX was mediated through a reduction of *de novo* thymidylate synthesis. These were:

1. The continued generally unimpaired incorporation of ^{14}C formate into RNA purines after treatment, Table 1.

2. The ^{14}C labelling of individual bases isolated from tissue DNA. Table 3 shows ^{14}C formate incorporation into bases 12 hr after

Table 3. *^{14}C labelled bases* 12 *hours after* 14*mg/kg MTX*

	Adenine	Guanine	Thymine
Spleen	20(29)	30(9)	4(62)
Tumour	78(37)	87(11)	22(52)
Gut	99(34)	91(19)	45(47)
BM	72(30)	79(19)	28(51)

Values represent percentage of control values. Results were calculated as dis/min associated with base/μg DNA. Figures in parentheses represent the percentage of ^{14}C associated with each individual base; i.e., 62% of ^{14}C isolated from the spleen extract was thymine.

a dose of 14 mg/kg MTX. These results indicate a substantial reduction of labelled thymine with a lesser effect upon adenine and guanine. Approximately 50% of the ^{14}C from formate was incorporated into DNA as thymine. Whilst there were smaller reductions in the level of labelled purines, the large reduction in labelled thymine probably accounts for the toxic effect of MTX.

3. A comparison of the fractional incorporation of ^{14}C formate and ^{3}H UdR (Fig. 2–5) shows that formate incorporation was not reduced as much as UdR in any of the tissues. Deoxyuridine is solely a *de novo* thymidine precursor, whereas formate is involved both in this pathway and in *de novo* purine synthesis. Hence, the difference between the uptake of the two precursors may represent continued *de novo* purine incorporation.

Biochemically this phenomenon has already been observed [29] and can be explained by the fact that the formation of the purine skeleton results in the regeneration of tetrahydrofolic acid which can recycle. Thymidylate synthesis, on the other hand, involves the conversion of the intermediate N^5, N^{10}) methylene-tetrahydrofolic acid ($h^{5-10}FH_4$) to dihydrofolic acid, and the reduction of the latter to the tetrahydro form is inhibited by the antifolate.

Because the cytotoxicity of MTX appeared to be mediated through prevention of *de novo* thymidylate production, the relative importance of an alternative thymidylate *salvage* synthesis had to be considered. In rats treated with MTX the possibility existed of a compensatory effect,

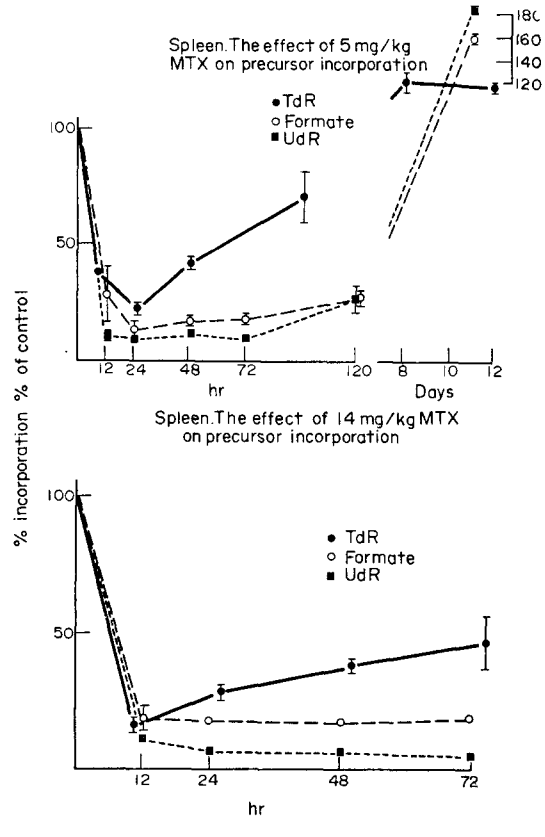

Fig. 2. *The effects of* 5 *mg and* 14 *mg/kg MTX on DNA precursor uptake in spleens of tumour bearing rats. The ordinate represents F.I. values as percent of controls. The abscissa is the time after a single dose of MTX. Each point is the mean of* 4 *experiments. Error bars represent* $\pm$ *SEM.*

or "rescue", of certain tissues with salvaged thymidylate via the thymidine kinase pathway. The uptakes of both *de novo* and *salvage* precursors are shown in Fig. 2–5 expressed as a percent of control. By this method, it was possible to compare the depression and subsequent recovery of each tissue with regard to possible differences between normal and tumour tissues.

Although not a dose-limiting tissue, the spleen has shown some interesting responses

to MTX (Fig. 2). A depression in spleen weight after MTX treatment was followed by a recovery and overshoot to 3–5 times the control weight by 10 days. This phenomenon was closely mirrored by DNA precursor uptake. Whilst the *de novo* precursor incorporations appeared to have increased more than the *salvage* at 10 days in terms of the control values for each, (cf. Table 2) the absolute increases were similar. Microscopy of the stimulated spleen has revealed an increase in the number of giant cells at 10 days. Such

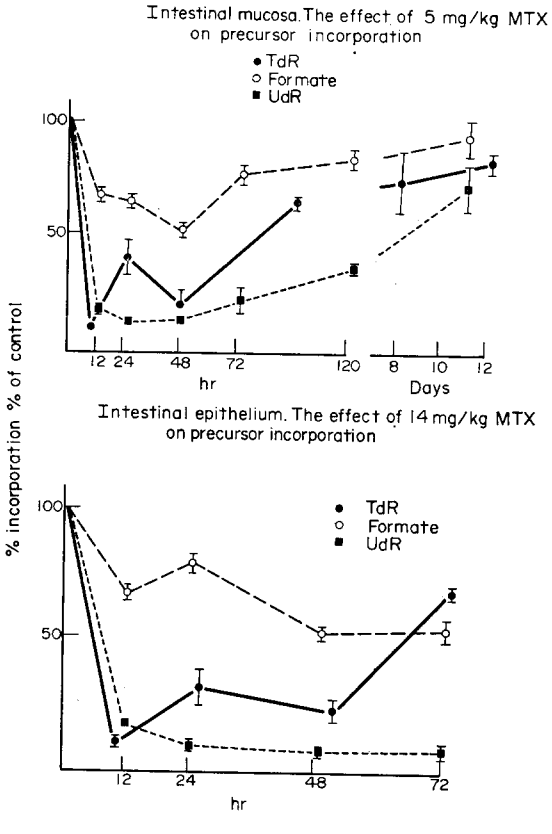

Fig. 3. The effects of 5 mg and 14 mg/kg MTX on DNA precursor uptake in jejunal mucosa. The ordinate represents F.I. values as percent of controls. The abscissa is the time after a single dose of MTX. Each point is the mean of 4 experiments. Error bars represent ± SEM.

spond to gut failure [9, 30]. Thus, the gut appeared to be the dose limiting tissue with respect to MTX treatment. Figure 3 showed that breakdown of gut function was reflected in the inhibition of precursor uptake. The apparent lack of inhibition of formate incorporation has already been attributed to continued *de novo* purine synthesis. Incorporation of UdR was reduced to 10% or less of control by 24 hr. At the higher dose there was no recovery by 3 days. At the lower dose UdR incorporation was approaching control levels

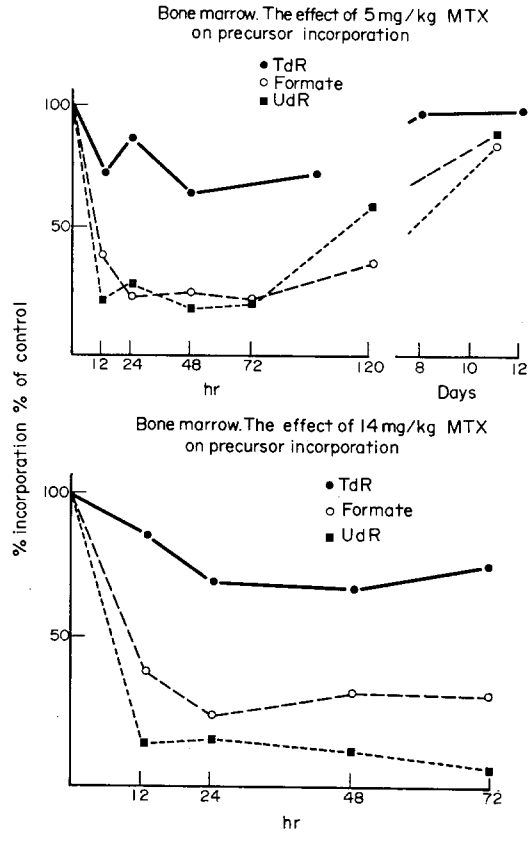

Fig. 4. The effects of 5 mg and 14 mg/kg MTX on DNA precursor uptake in bone marrow. The ordinate represents F.I. values as percent of controls. The abscissa is the time after a single dose of MTX. Each point is the mean of 4 experiments. Error bars represent ± SEM.

increased splenic activity may be linked with increased lymphocyte production, which may have been a direct drug effect, or a response to, infection. Macroscopic and microscopic pyorrhoeic inflammations have been observed suggesting that the latter may be the more likely cause of such activity.

Toxicity studies in the August rat have shown that animals receiving lethal doses of MTX die between 3 and 5 days after treatment. Animals which survive this period, recover completely. This period of time has been shown to correby 11 days. MTX should not have a direct effect upon TdR incorporation. Therefore, the marked reduction seen at both doses was attributed to resultant cell death. In other words the restriction of *de novo* thymine supply was sufficient to prevent compensation by *salvage* synthesis, resulting in cell toxicity and the reduction in scheduled DNA synthesis.

Microscopic examination of the jejunum after a single lethal dose of MTX has shown a

massive destruction of vullus and crypt integrity at 3 days (plates1–4). Gross macroscopic swellings of the Peyers patches were visible and a section through such a swelling revealed an unusual protective effect. Crypt and villi in the immediate area of the swollen lymph nodes were intact and in some cases slightly hyperplastic. Why such "pockets" of actively dividing cells should occur was not clear. Supply of drug via the blood stream may have been limited, however blood vessels in the area appeared to be healthy. A further

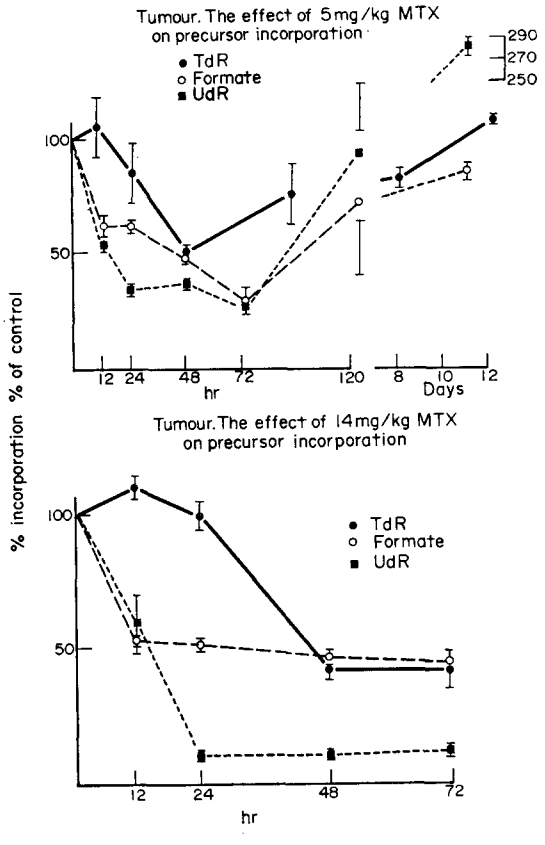

Fig. 5. The effects of 5 mg and 14 mg/kg MTX on DNA precursor uptake in tumour. The ordinate represents F.I. values as percent of controls. The abscissa is the time after a single dose of MTX. Each point is the mean of 6 experiments. Error bars represent ± SEM.

possibility was that, in some obscure manner, the lymphocytes "mopped up" the MTX before it reached the mucosal cells. This may in turn, have stimulated lymphocyte production and thus resulted in the observed swellings. Finally, it is possible that there were mucosal cells resistant to MTX and these continued to divide even after therapy. These isolated areas of mucosa were obviously insufficient to allow normal gut function, since the animals still died between 3 and 5 days.

Precursor uptakes in the bone marrow (Fig. 4) showed a pattern similar to gut for formate and UdR, but dissimilar for TdR. Both the *de novo* precursors were depressed to around 20% of control at both doses.

TdR *salvage* remained around 80% of control. This continued level of *salvage* synthesis may have enabled a partial by-pass of the MTX induced *de novo* block and thus accounted for the reduced toxicity of MTX to bone marrow. Rabbit bone marrow has been shown to lack the intrinsic enzymes needed for *de novo* purine synthesis [31]. If the same is true for rat bone marrow, the ^{14}C-formate uptake will be a measure of blood-borne purines. Presumably these will be supplied by the liver which has been shown to incorporate ^{14}C-formate into purine nucleotides [32]. It was not possible, in this study, to estimate the proportions of formed purine nucleotides derived from liver and from synthesis *in situ*, however, it is possible that a balance between the two sources exists in all tissues.

In contrast to the three tissues already discussed, the tumour (Fig. 5) showed no initial suppression of TdR incorporation. In fact, at 14 mg/kg MTX a slight increase in *salvage* synthesis was observed. This is in common with the results of Tattersall and Harrap [33] who observed a steady rise in TdR incorporation in L5178Y cells up to 8 hr after a dose of MTX. In contrast both UdR and formate incorporation were reduced. Additionally, this dose increased the level of acid soluble ^{3}H with TdR, whilst concomitantly reducing that of UdR (Table 4). The MTX induced reduction in *de novo* thymidylate must, in effect, reduce the levels of intracellular TMP. The alternative *salvage* thymidylate pathway is under cellular regulation. Such a reduction in the intracellular levels of TMP results in increased thymidine kinase activity [34] and therefore a rise in incorporation of ^{3}H-TdR. This feedback mechanism may operate at different intracellular TMP levels in different tissues. Thus, the tumour may be stimulated to "turn on" to *salvage* synthesis when levels of TMP are not at as critical a level as those of, for example, intestinal mucosa. The extent and rate of enzyme induction may be critical to the recovery of the tissue and these factors may have accounted for the extra gut toxicity in these rats. In the tumour, at both 5 and 14 mg/kg there was a drop in TdR incorporation at 48 hr. This would suggest that the early burst of *salvage* synthesis was insufficient to compensate for the reduced *de novo* supply and ultimately this led to cell

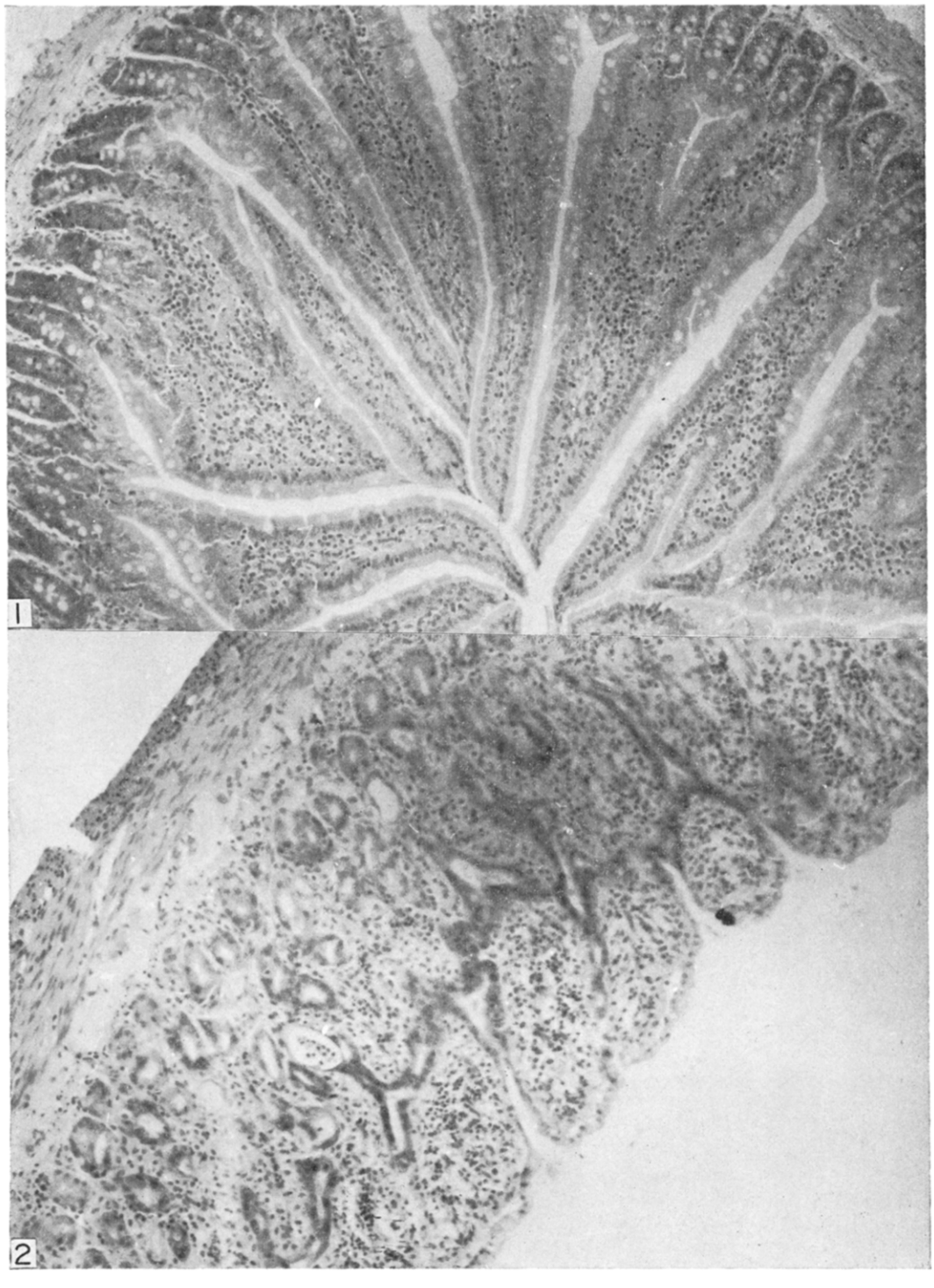

PLATE 1. *Low power cross section of untreated jejunum, showing normal crypt and villus structure.*

PLATE 2. *Low power cross section of jejunum, 3 days after a dose of 14 mg/kg MTX. Gross morphological abnormalities include atritic, vacuolised crypts denuded of cells. Bottom left centre is a clear example of a vacuolised crypt containing dead cells and lymphocytes; as a result of the crypt abnormalities, the villi are flattened, fused and irregularly shaped.*

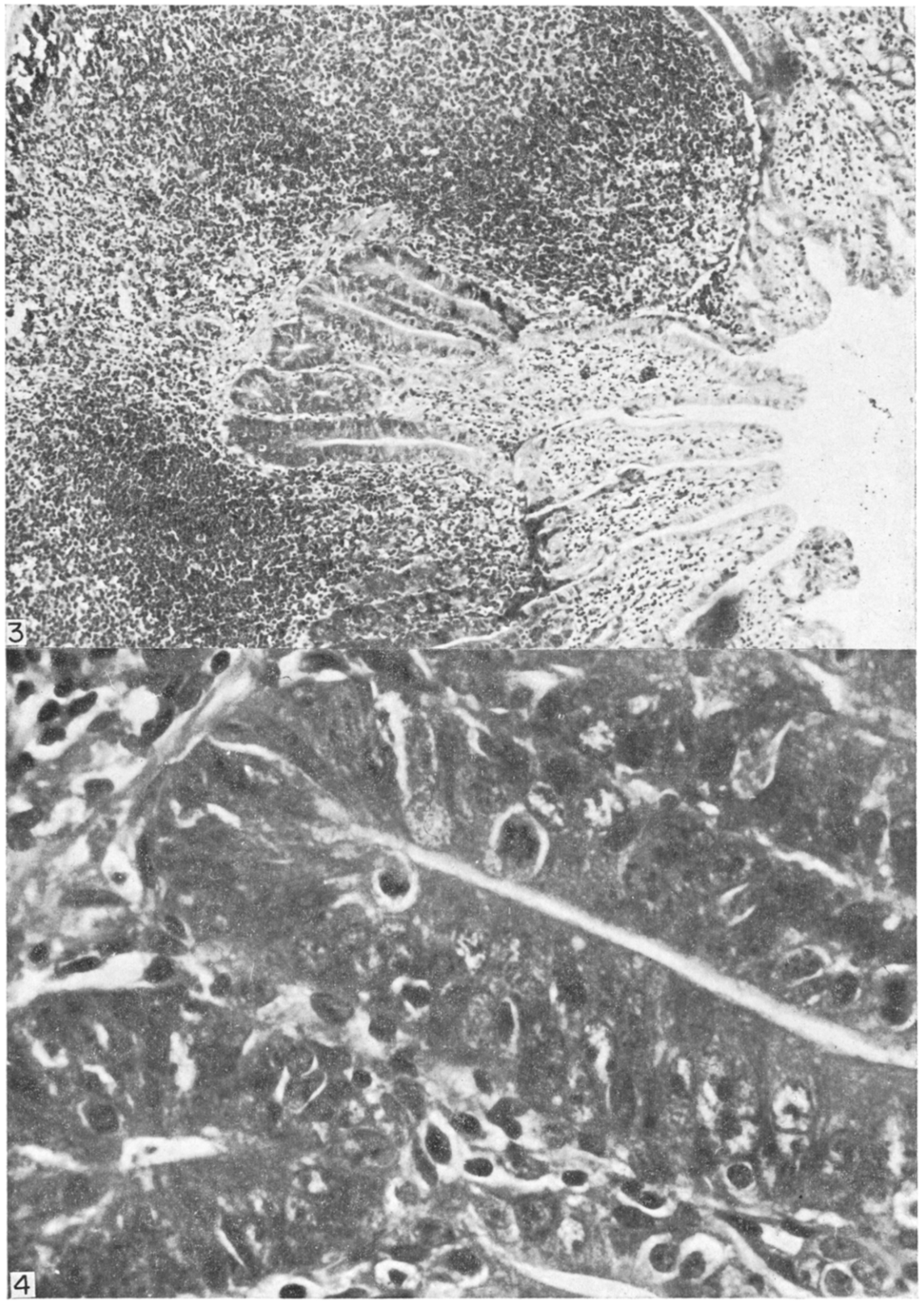

PLATE 3. *Low power cross section of the same section as* [2] *which includes the Peyers patch area. The two large lymph nodes show the dark staining mature lymphocytes which are pushed outward from the lighter stained, actively dividing core. Between the nodes are hyperplastic crypts which, uncommon with the remainder of the section, are undergoing mitoses* [4]. *The crypts are approximately* 2–3 *times as long as those of controls. The villi associated with these crypts appear fairly regular in structure.*

PLATE 4. *High power view of part of crypt shown in* [3]. *Five definite mitoses can be seen in this view.*

Table 4. Levels of acid soluble ^{3}H *after 14mg/kg MTX*

	TdR				UdR			
	Spleen	Tumour	Gut	Liver	Spleen	Tumour	Gut	Liver
Control	83	101	131	825	237	239	232	521
12H	195	162	206	1521	169	167	213	416
24H	171	139	184	1327	198	198	227	389
48H	165	194	185	832	210	188	218	452
72H	91	137	240	522	252	227	260	506

Results are expressed as ^{3}H dis/min per mg wet wt. tissue. Each value is the mean of at least 4 experiments. Standard errors $< 10\%$.

death. It may be possible, by extending these studies, to gain a quantitative relationship between the suppression of *de novo* synthesis and the requisite compensatory *salvage*, by relating these parameters to gut and tumour toxicity plus animal survival.

The increase in UdR incorporation to three times control level, 10 days after 5 mg/kg MTX, was unexpected. There was no decrease in acid soluble ^{3}H levels which could have suggested intracellular pool changes. A possible explanation was that the drug selected a population of tumour cells high in thymidylate synthetase activity and thus, by 11 days the tumour had a population of cells more adapted to utilising UdR. Although this should have caused a concurrent increase in formate incorporation. Alternatively the drug may have reduced the level of UdR catabolising enzymes, with the result that more is converted to thymidylate.

The tumour volume responses shown in Fig. 6 correlated well with the biochemical data. The volume continued to increase for 2 days after treatment. This fact concurred with results obtained using cyclophosphamide [35] and *cis*-dichlorodiammino platinum [36]. After this, volumes regressed for 4–6 days, before resuming exponential growth. This period of volume regression correlated with the reduction in DNA precursor incorporation. By day 6 post-therapy near control levels of precursor incorporation had been reached.

In conclusion, the therapeutic efficacy of MTX toward tumours in the August rat was reduced because of extreme toxicity to the intestinal mucosa. The reduction of *de novo* thymidylate to the bone marrow and tumour was apparently less toxic. These tissues were more efficient at overcoming this block with an alternative *salvage* pathway.

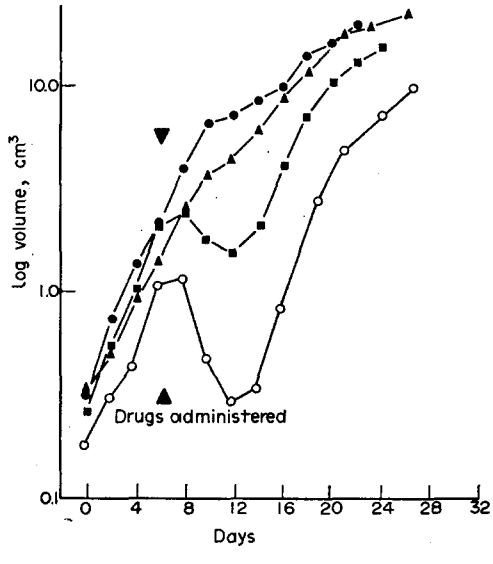

Fig. 6. Volume response of BICR A15 rat tumour after various doses of MTX. The ordinate is a log plot of tumour volume. The abscissa is time in days. MTX was administered 6 days after tumours were first palpable which was 8 days after serial transplant

● *Control*
▲ *Pair fed control*
■ 5 *mg/kg MTX*
○ 10 *mg/kg MTX*

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Letter to the Editor

Magnesium Concentration Changes in Blood and in "Target" Tissue During Carcinogenesis*

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THE HEPATOCARCINOGENESIS induced by 4-dimethylaminoazobenzene (DAB) provokes changes in cell membrane permeability which are reflected by increased influx of extracellular cations [1].

In order to establish if there is a relationship between the ionic contents of blood and liver tissues during the carcinogenesis, samples of both tissues were analyzed for their mineral content. For this purpose male Wistar rats (100–110 g of body weight) were fed a basal semisynthetic diet supplemented with 0·06% of DAB. The animals were allowed to eat *ad libitum* and they were killed after different periods of feeding. Rats without DAB were used as controls. Before the sacrifice blood samples were drawn by cardiac punction. After mineralization by ashing at 700°C and dissolution in 1 N HCl, calcium, magnesium, sodium and potassium were determined by Atomic Absorption Spectrometry. Total phosphorus was assayed by means of the 4-aminonaphtol reagent [2].

The results showed that DAB-feeding provokes an immediate increase of magnesium and phosphorus in total blood. At the beginning of the period of development of liver tumor (5–10 months of DAB-feeding) those values showed a slight decrease and they remained all the time almost constant but significantly higher than in the control group (Fig. 1). The surviving animals presented a higher than in the control group magnesium concentration while phosphorus decreased

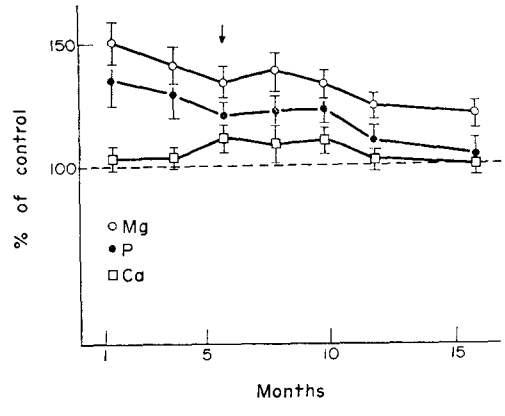

Fig. 1. *Magnesium, calcium and phosphorus in whole blood of animals fed* 4-*dimethylaminoazobenzene. The values are the percent of the concentration corresponding to the control group and the mean value* ± *S.E. of a* 10-*animal group. The arrow indicates the onset of macroscopically observable liver tumors.*

sharply to almost the control values. Blood calcium did not show significant changes and only during the period of development of tumor a noticeable increase was observed. In tumor-bearing animals blood magnesium and phosphorus were significantly increased with respect to the controls. The increase of blood magnesium (approximately 50%) accompanies a significant decrease of this element in neoplastic liver (approximately 30%). On the other hand, the very high increases of calcium and sodium in tumor issue did not show a correlation with their contents in

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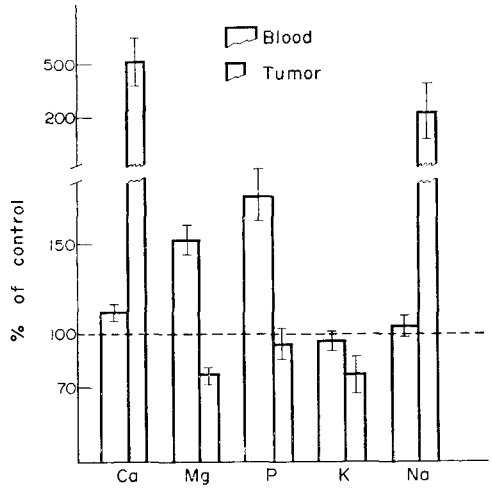

Fig. 2. Magnesium, calcium, sodium, potassium and phosphorus in whole blood and in liver tumor tissue from tumor-bearing animals. The values of liver tumor are compared with the values corresponding to normal liver tissue from the animals of the control group. The values are the percentage of the concentration corresponding to the control group and the mean value ± S.E. of a 20-animal group.

blood (Fig. 2). The electrophoresis of blood serum showed that in tumor-bearing animals there is a slight increase (9·5%) of α-globulins accompanied by a decrease of β- (12%) and γ-globulins (19%), and at the same time the total serum protein was lower (14%).

The high blood magnesium right after starting DAB-feeding seems to be related to its decrease in liver tissue observed at this time of the carcinogenesis [3]. The fact that the ratio intracellular magnesium to extracellular magnesium in liver is approximately 20·0 while the same ratio for calcium is 0·55 [4], and that liver is one of the tissues with the highest concentration of magnesium [5], may explain the increase of blood magnesium as the result of a lost and/or an exclusion of this element from liver tissue. On the other hand, it seems evident that the differences of magnitude between the blood pool of calcium and its content in liver can explain the observation that modifications of its concentration in liver tissue are not reflected on its blood content as it is in the case of magnesium.

The absence of significant differences in the protein composition of blood serum indicates that they do not seem to play an important role in the modification of magnesium and calcium homeostasis during carcinogenesis by DAB.

These experimental results point out that in this type of chemical carcinogenesis there are changes in homeostasis which appear to be related to modifications of the balance of cations in the target tissue. In addition, these observations are consistent with the concept of changed cell membrane permeability as one of the factors triggering the development of neoplasia [3].

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Letter to the Editor

Heterogeneity and Specificity of Circulating Carcinoembryonic Antigen*

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CARCINOEMBRYONIC antigen (CEA), first described by Gold and Freedman [1], is a tumour associated macromolecular glycoprotein found in the cellular membrane of digestive system epithelia. While its presence in high concentration in serum is indicative of metastatic cancer [2], low to moderate levels are associated with both primary cancer and a number of non-malignant conditions particularly inflammatory diseases [2, 3]. This has indicated a need for greater specificity in the CEA assay if it is to be used diagnostically or to monitor less advanced cancer. In our approach to this problem we have applied concanavalin A affinity fractionation to a study of CEA in serum and we suggest that two forms of CEA may be of diagnostic significance.

Earlier work in this [4–6] and other [7, 8] laboratories has shown that CEA, prepared essentially by the method of Coligan *et al.* [9] from metastatic colonic tumour, can be fractionated by affinity chromatography on Con A–Sepharose. An unbound fraction 1 and three bound fractions designated 2A, 2B and 3 can be successively eluted from the column using sodium acetate buffer (0·1 M pH 6 containing 1 M NaCl), sodium borate/phosphate buffer (0·1 M pH 6), 2% methyl glucoside and 10% methyl glucoside [6].

In our approach reported here in preliminary form, we have fractionated patients' sera on Con A–Sepharose and assayed conventionally the CEA recovered in the fractions obtained. The carbohydrate binding site of Con A appears to be directed primarily towards unmodified hydroxyl groups at the C-3, C-4 and C-6 positions of α-D-mannose and α-D-*N* acetylglucosamine in the outer chains of CEA [10]. It follows, therefore, that these chains must differ chemically in the CEA fractions. Perchloric acid extracts of serum pools containing a total of at least 100 ng of CEA were dialyzed for 3 days against tap water, concentrated to 5 ml by ultrafiltration using an Amicon PM 10 membrane and the CEA measured by our conventional assay. The extract was then separated into 4 fractions (1, 2A, 2B and 3) as previously described [6]. Each fraction was collected, dialyzed against tap water to remove buffer salts and methyl glucoside and concentrated to known volumes (2–8 ml depending on the total CEA applied to the column). The CEA in each fraction was determined by our conventional assay and the results expressed as μg CEA/10 l of original serum. As a control, sera known to contain less than 2·5 μg CEA/l was fractionated in the same way.

The results, shown in the table, are classified into the three disease groups; (a) local cancer including some cases with local or regional lymph node involvement and where the routine assay value is moderately elevated, (b) cases with advanced cancer involving distant nodes or liver metastases and (c) non-malignant diseases where the serum CEA value was raised. In group (a) CEA was detectable in significant amounts in fraction 2B and as expected the levels were moderately raised. CEA was below detectable levels in fractions 1 and 2A and, with the exception of one experiment where the value was low, CEA was also undetectable in fraction 3. This is in contrast to group (b) where the CEA values were much higher. In this case small amounts of CEA were detected in fractions 1 and 2A. However, since this may be a quantitative effect where the CEA values in these two fractions may be raised to significant levels due to the higher concentration of CEA, no diagnostic significance can be attributed to this finding. Most of the CEA in

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Table 1. *CEA (μg/10 l.)* in Con A fractions obtained from serum pools from three groups of diseases*

	No. in pool	1	2A	2B	3	% Recovery †
Group (a) Local Cancer						
Colon	4	‡		73		70
Gynaecological	11			31		
Gastric	40			15		
Colon	7			16		49
Colon	8			86	13	73
Group (b) Advanced Cancer						
Colon	8	67		>1230	>720	
Colo-rectal	9			>225	>1000	
Colon	10	73	107	396	515	52
Rectal	8	46	79	570	478	58
Non-colonic	34	+		++	+++	
Gastric	13	18	33	166	101	50
Colon	10	34	36	605	357	46
Thyroid	2			40	70	
Pancreas	6			44	46	54
Group (c) Non Malignant						
Rheumatoid arthritis	13		9	15	5	80
Rheumatoid arthritis	12				12	60
Crohn's Disease	10				32	

*The CEA values are lower than those assayed in the original serum owing to loss of CEA on extraction with perchloric acid, loss of CEA which is irretrievably bound to Con A–Sepharose and experimental loss of CEA during concentration of fractions.

†% recovery calculated as CEA recovered from column × 100/CEA applied.

‡CEA in the dialysed fraction after concentration less than 5 μg/l.

the sera of group (b) was eluted in fractions 2B and 3 and the relative levels in these two fractions suggest a distinct qualitative difference in the elution pattern compared to sera from group (a). In the case of sera from the non-malignant group (c), it appears from the limited data so far available that CEA is elevated in fraction 3.

Our results indicate that the chemical differences which exist between the different Con A fractions [5, 10] may be implicated in the specificity of the CEA molecule for cancer. It appears that one particular form of CEA may be associated with both early and advanced cancer. Another form which binds more strongly to Con A, appears to be associated with metabolic changes found both in advanced cancer and in certain non-malignant inflammatory diseases. Our results also suggest that certain CEA molecules have a cancer specific grouping which is distinct from the dominant immunogenic group.

Conventional assays and modified assays based on labelled CEA fractions, although useful for follow-up studies and detecting recurrent cancer [11, 12], are severely limited in specificity due to the cross-reactive nature of the antibody. Our studies suggest that it may be possible to design more specific CEA assays. One possibility would be to incorporate a separation stage prior to a conventional assay, although the ease of handling large numbers of specimens on a routine basis would be an important factor in its acceptibility. At the same time the search for antisera with high specificity for particular CEA fractions as suggested by Vrba *et al.* [13] also remains appropriate.

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Abstracts from XI Paterson Symposium

Book Reviews

G. Blackledge: Oncologic medicine—(Edited by A. I. Sutnick & P. F. Enstrom) University Park Press, Baltimore (1976).

M. Fox and D. Scott: Human tumours in short term culture: Techniques and clinical applications (Edited by P. P. Denby.) Academic Press (1976).

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P. BENTVELZEN and J. BRINKHOF

Organ distribution of exogenous murine mammary tumour virus as determined by bioassay.

P. CREEMERS and P. BENTVELZEN

Cellular immunity to the mammary tumour virus in mice bearing primary mammary tumours.

M. DE BRABANDER, G. GEUENS, M. BORGERS, R. VAN DE VIERE, F. THONE, J. DE CREE, F. AERTS and L. DESPLENTER

The effects of R17934 (NSC 238159): A new antimicrotubular substance on the ultrastructure of neoplastic cells *in vivo*.

M. SZEKERKE and J. S. DRISCOLL

The use of macromolecules as carriers of antitumor drugs.

J. LINKS, O. TOL, J. CALAFAT and F. BUIJS

Biological activities of murine mammary tumour virus *in vitro*. Increased macromolecular syntheses in mouse and hamster kidney cells; production of B- and C-particles in the mouse cells.

A. MCBRIDE and J. J. FENNELLY

Immunological depletion contributing to familial Hodgkin's disease.

C. J. H. VAN DE VELDE, L. M. VAN PUTTEN and A. ZWAVELING

A new metastasizing mammary carcinoma model in mice: model characteristics and applications.

R. C. JACKSON and D. NIETHAMMER

Acquired methotrexate resistance in lymphoblasts, resulting from altered kinetic properties of dihydrofolate reductase.

J. LINKS, J. CALAFAT, F. BUIJS and O. TOL

Simultaneous chemical induction of MTV and MLV.

R. BASSLEER and F. DE PAERMENTIER

Cytological and cytochemical analysis of two mouse cancer cell lines. Caryotype, number of nucleoli, DNA RNA and protein contents.

G. F. ROWLAND

Effective antitumour conjugates of alkylating drug and antibody using dextran as the intermediate carrier.

E. N. COLE, R. A. SELLWOOD, P. C. ENGLAND and K. GRIFFITHS

Serum prolactin concentrations in benign breast disease throughout the menstrual cycle.

A. RAZ, M. INBAR and R. GOLDMAN

A differential interaction *in vivo* of mouse macrophages with normal lymphocytes and malignant lymphoma cells.

E.O.R.J.C. International Antimicrobial Therapy Project Group

Protocol for a co-operative trial of empirical antibiotic treatment and early granulocyte transfusions in febrile neutropenic patients.

Gunhild Lange Wantzin and Sven-Aage Killman

Nuclear labelling of leukaemic blast cells with tritiated thymidine triphosphate after daunomycin.

A. P. Anzil, D. Stavrou, K. Blinzinger and U. Osterkamp

Interactions of concanavlain A with cell surfaces of normal and tumor rat glial cells monitored by agglutination and cytochemical detection.

A. Tagliabue, N. Polentarutti, A. Vecchi, A. Mantovani and F. Spreafico

Combination chemo-immunotherapy with adriamycin in experimental tumor systems.

E. N. Cole, P. C. England, R. A. Sellwood and K. Griffiths

Serum prolactin concentrations throughout the menstrual cycle of normal women and patients with recent breast cancer.

Rashida A. Karmali and D. F. Horrobin

Effects of prolactin and suppression of prolactin secretion on experimental tumours of lung and muscle in mice.

Europ. J. Cancer Vol. 13, p. 302. Pergamon Press 1977. Printed in Great Britain

Medical Oncology Society

INTRODUCTION

ONCOLOGY comprises several disciplines which include surgery, radiology and medical oncology, whose objective is to eradicate cancer.

Medical oncology poses several problems today for which solutions are not being found and applied quickly enough in the interest of patients.

Medical oncology develops along a geometrical curve. For ten years, in the strict sense, it has cured the following conditions: acute lymphoid leukaemia, Hodgkin's Disease and a certain number of lymphosarcomas and, for three years, it has appeared to be able to prevent the occurrence of metastases of six solid tumours: breast, colon, and stomach cancers and osteosarcomas by systemic post-operative chemotherapy, and cancer of the lung and melanomas by systemic post-operative immunotherapy.

This work has not yielded 100% results: intensive research has to be carried out if we are ever going to approach this goal.

These results require rapid mutual information amongst medical oncologists, their co-operation and an appropriate status for them, the preparation by them of younger colleagues, and the adaptation of the best logistics to their activities.

The creation of the Medical Oncology Society was vital to find and speed-up solutions to these problems.

The number of participants, the range of nationalities, and the quality of work presented at its first annual congress confirms the need for such a society and augurs well for the success of this venture.

G. MATHE,
President

Europ. J. Cancer Vol. 13, p. 303. Pergamon Press 1977. Printed in Great Britain

La "Société de Médecine Interne Cancérologique"

La cancérologie met en oeuvre plusieurs disciplines technologiques, dont la chirurgie, la radiologie, et la médecine interne cancérologique, qui coopèrent dans le traitement de la plupart des tumeurs.

La médecine interne cancérologique pose aujourd'hui plusieurs problèmes auxquels les solutions ne sont pas apportées aussi rapidement que l'exige l'intérêt des malades.

Cette discipline se développe à une vitesse pour le moins géométrique; depuis dix ans, elle permet de guérir *stricto sensu* des patients porteurs de cancers disséminés d'emblée: la leucémie aiguë lymphoide, la maladie de Hodgkin, certains lymphosarcomes; depuis trois ans, elle promet de prévenir les métastases de 6 tumeurs solides au moins: les cancers du sein, du colon, de l'estomac, et l'ostéosarcome, par la chimiothérapie post-opératoire systématique, les cancers du poumon et les mélanomes par l'immunothérapie post-opératoire systématique.

Nous n'obtenons pas 100% de résultats positifs; des travaux intensifs doivent être menés pour approcher ce taux.

Ces travaux demandent une information mutuelle rapide des médecins internistes cancérologues, leur coopération, l'affirmation de leur rôle, la formation, par eux, de jeunes collègues, et l'adaptation de la logistique cancérologique aux activités de cette discipline.

La création de la Société de Médecine Interne Cancérologique était indispensable pour apporter et accélérer les solutions à ces problèmes.

Son premier Congrès annuel, le nombre de ses participants, la multiplicité de leur nationalité, et la qualité de ses travaux, confirme le besoin de cette Société et augure le succès de cette entreprise.

Europ. J. Cancer Vol. 13, pp. 305–308. Pergamon Press 1977. Printed in Great Britain

Papers presented at the "First Congress of the Medical Oncology Society, Nice"*

I. IMMUNE EXPLORATION

D. BELPOMME, N. LELARGE, R. JOSEPH and G. MATHE

An immunological classification of leukemias and non-Hodgkin's hematosarcomas based on cell membrane markers with special reference to null cell disorders.

J. BERTOGLIO, D. GERLIER, A. BOURGOUIN, K. GRONNEBERG and J. F. DORE

Increase in E. active rosette forming lymphocytes in melanoma patients treated with BCG.

H. G. BOTTO, H. GAUTHIER, P. POUILLART, M. BRULEY-ROSSET and P. HUGUENIN

Correlation of delayed hypersensitivity and leukocyte migration inhibition factor in the serum of cancer patients.

M. BRULEY-ROSSET, H. G. BOTTO and A. GOUTNER

Serum migration inhibitory activity in patients with infectious diseases and various neoplasia.

P. CAPPELAERE and A. M. GRANIER

Serum immunoglobulins and C3 levels in cancerous patients.

Y. CARCASSONNE, G. MEYER and R. FAVRE

Surface immunofluorescence in Hodgkin's disease.

PH. CHOLLET, J. CHASSAGNE, C. THIERRY, B. SAUVEZIE, B. SERROU and R. PLAGNE

Isolation and electrophoretic mobility of three lymphoid populations in normal human blood.

D. DANTCHEV

Revised semiology of the different mononuclear cells under scanning electron microscopy.

A. J. S. DAVIES

A short essay on B and T lymphocytes.

A. DESPLACES, A. BOURGUIGNAT, A. GENTILE, P. GUERIN and R. T. SARACINO

Immunological study of axillary lymph-nodes in patients with breast cancer.

J. F. DORE, C. GUIBOUT, J. BERTOGLIO and A. LIABEUF

Cytotoxic antibodies to human leukaemia cells in normal human sera.

J. M. LANG, FR. OBERLING, S. MAYER

T lymphocyte count in untreated lymphoproliferative disorders.

O. LEES

Immunoperoxidase method of B lymphocyte recognition.

*Not all of the papers presented were selected for publication in the Journal.

II. NATURAL STORY OF CANCER
CLINICAL AND BIOLOGICAL EXPLORATION OF PATIENTS

III. CANCER CHEMOTHERAPY

IV. IMMUNOTHERAPY

V. HORMONOTHERAPY—STRATEGY AND SYMPTOMATIC TREATMENT

Europ. J. Cancer Vol. 13, pp. 311–319. Pergamon Press 1977. Printed in Great Britain

An Immunological Classification of Leukemias and Non Hodgkin's Hematosarcomas Based on T and B Cell Membrane Markers with Special Reference to Null "Cell" Disorders

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THE IDENTIFICATION, several years ago, of membrane markers on the mononuclear cell population of both animals [1] and humans [2–9] has permitted differentiation of this population into T (thymodependent) and B (thymoindependent) lymphocytes and a third cell category which includes monocytes [10, 11].

The presence of these markers on normal as well as on neoplastic cells of lymphomonocytoid proliferative disorders has led to effort to reclassify human hematologic malignancies into different immunological categories [4, 9, 12–16].

The clinical usefulness of this new classification still remains uncertain, but it is evident from these studies that new concepts concerning the mechanism of cancerogenesis and the origin of these neoplastic cell proliferations have emerged [4, 12, 16, 17].

In previous papers we have shown that acute lymphoid leukemia (ALL) is a T cell disorder in 25% of cases, while chronic lymphoid leukemia (CLL) is a B cell disorder in 90% [4, 12].

We have shown in addition, that while non-Hodgkin's hematosarcomas (lymphomas) are B cell diseases in the majority of cases, they are of the T cell type in some of them [14, 18, 19].

We have, in fact, emphasized that by using these markers in ALL, as well as in non-Hodgkin's hematosarcomas, there were neoplasias which could not be typed as T or B, and that those proliferations may be related to so-called null cell disorders [4, 14, 18, 19].

These findings, however, need a critical evaluation since (a) technical problems are still encountered in the detection of lymphocyte membrane markers and since (b) results published in the literature dealing with so-called null cell disorders most often concern limited series of cases [20, 21].

The purpose of this paper is firstly, to assess the value of the immunological methods available for the characterisation of normal human mononuclear cells and, secondly, to report our findings, based on these techniques, in an attempt to classify 150 cases of mononuclear cell proliferative disorders. This study will evaluate the frequency of so-called null cell diseases, and will give a clinical summary of these cases.

MATERIAL AND METHODS

1. *Immunological methods*

Table 1 shows different membrane markers and the tests used to characterize T and B lymphocytes and monocytes. Table 2 presents the membrane phenotypes of the different normal human mononuclear cells.

Details of the test procedures used have already been reported [4].

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Table 1. T and B cell membrane markers of normal human lymphocytes and monocytes

Marker	Test	T	B	Third category*
SRBC receptors	E rosettes	+	0	0
MRBC receptors	E rosettes	0	(+)	0
Fc ag-IgG receptors	EA rosettes	(+)?	(+)?	(+)
Fc Ig receptors	Immunofluorescence with heat aggregated Ig	0	+	+(?)
Activated C_3 (d or b) receptors	EAC rosettes	(+)?	(+)?	(+)
Membrane Ig	Membrane immuno-fluorescence	0	+	0
Specific T, B hetero antigen(s)	Cytotoxicity or immunofluorescence	+, 0	0, +	0, 0
EBV receptors	Rosettes	0	(+)	0

S.R.B.C.: Sheep red blood cell.
M.R.B.C.: Murine red blood cell.
Fc ag-IgG: Fc receptors for antigen–IgG. antibody complexes
Fc–Ig: Fc receptors for immunoglobulins
Activated C'_3: Activated C'_3 complement receptors
EBV receptors: Receptors for Epstein–Barr virus
E rosettes: Spontaneous rosettes
EA rosettes: Binding of erythrocytes sensitized with anti-erythrocyte antibodies
EAC rosettes: Binding of erythrocytes sensitized with and complement.

*Non T non B mononuclear cells including monocytes.
( ) On some cells.

Table 2. Membrane phenotypes of human T and B lymphocytes and monocytes

T CELL	= mIg(−), Fc agIg*(?), Fc(−), C_3(?), E(+), Em(−) T ag(+) B ag(−)
B CELL	= mIg(+), Fc agIg(+), Fc(+), C_3b (+), C_3d†(+), E(−), Em(+), T ag(−), B ag(+)
"MONOCYTE"	= mIg(−), Fc agIg(+), Fc(−), C_3b (+), C_3d†(+), E(−), Em (?), T ag(−), B ag(−)

*Present on educated T cells (?); possible characteristic of K cells (?)
†Involved in the immune adherence phenomenon.

mIg Membrane immunoglobulins.
FcagIg Fc receptors for antigen Ig antibody complexes.
Fc Fc "receptors" for free immunoglobulins.
C'3 Activated C'3 complement receptors; two subvarieties C'_3b and C'_3d.
E Spontaneous rosette formation with sheep red blood cells.
Em Spontaneous rosette formation with murine red blood cells.
Tag Specific T heteroantigen(s).
Bag Specific B heteroantigen(s).

Table 3 gives the values obtained by these techniques for mononuclear cells from the peripheral blood, bone marrow and thymus of 50 normal subjects used as controls. This data concurs with that published in the report of a WHO/IARC sponsored workshop [22].

Although most of the techniques described here are now well standardized, the practice of these tests, as well as the interpretation of the results, needs critical evaluation. From Table 4 it can be seen that there are some difficulties:

(1) The formation of spontaneous E rosettes (E rosette test) with sheep red blood cells (SRBC) requires special attention since a minor technical modification may induce major variations in the results. From Fig. 1 it can be seen that there are at least 4 variable parameters involved in the test [14, 18].

(2) The detection of membrane immunoglobulins (mIg), should not be confused with the evidence of surface immunoglobulins (sIg). Only the former seems to be specific for B lymphocytes, since the latter (sIg) may be:

Table 3. T and B cell markers in normal peripheral blood, bone marrow and thymus

	ROSETTES (%)*						mIg*	
	E		EA		EAC		(%)	
Peripheral blood (50 normal subjects)	50†	25–80‡	10	5–15	15	5–25	23	10–40
Bone marrow (3 normal subjects)	22	16–28	9	5–14	44	36–53	30	
Thymus (6 normal subjects)	97	83–100	ND	ND	ND	ND	3	2–5

*See Tables 1 and 2 for abbreviations. Results are expressed as the ratio of total number of positive cells on total number of mononuclear cells.
†Mean values.
‡Extreme values.
§Increased mean value are now obtained in our laboratory, by using a sensitized test (see Joseph 1976a, 1976b).

Table 4. Critical evaluation of the test procedures for detecting membrane markers

E Rosette tests	Variability of the test (differing techniques and results according to authors).
EA Rosette tests	EA receptors may be detected on some educated T lymphocytes, on some B cells, as well as on monocytes and K cells.
EAC Rosette tests	No good control distinguishing EA from EAC receptors.
Membrane immunofluorescence	Difficult to obtain specific antisera. Membrane Ig have to be distinguished from surface Ig. Ig monoclonicity is required for neoplastic B lymphoid cells.
Specific anti T or B heterosera	Difficult to obtain true specific hetero antisera. Immunofluorescence tests are not specific. Cytotoxicity tests do not allow correlation with morphological studies.
EBV membrane receptors	Difficulty due to the quality of the EBV+permanent cell line used in the test.

For abbreviations see Tables 1 and 2; for references, see text.

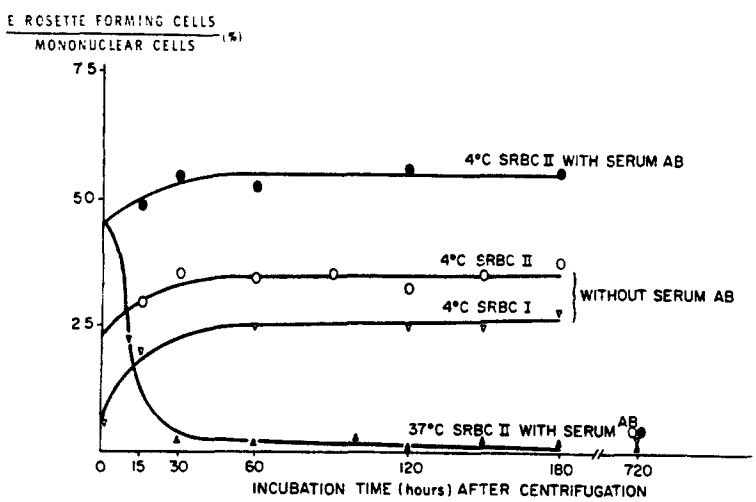

Fig. 1. Variable parameters involved in the E rosette test procedure. Experimental data concern the peripheral blood of six normal adult subjects. Note that the temperature, the quantity of SRBC, the incubation time and the eventual addition of human serum which has been heat decompleted and absorbed with SRBC, have to be considered.

(a) free immunoglobulins (Ig) (including antibodies) [14, 18], or (b) Aggregated Ig [23], coating the membrane of various cells, or (c) serum immune antigen-antibody complexes adhering to Fc receptors of monocytes [10], B lymphocytes, [4, 12, 13] of (activated?) T lymphocytes, or K cells [24, 25] or adhering to activated C'3 receptors of monocytes [10] or B lymphocytes [8, 26].

These various possibilities make the direct immunofluorescence test for mIg difficult to interpret and require proof that mIg are, in fact, synthetized by the B lymphocytes [9].

(3) Other difficulties concern the application of the immunologic techniques used for normal circulating cells, to neoplastic solid tissues: (a) there is a loss of cells during purification of mononuclear elements and thus possibly a cell selection; (b) presence of both normal and neoplastic cells in tumour tissues requires systematic morphological control to verify which cells possess the markers.

(4) Finally data on cell membrane typing using immunological criteria have to be correlated with other diagnostic methods such as the clinical picture, cytology, histology and conventional and scanning electron microscopy [19, 27–29]. These general considerations stress the need to use a battery of tests, rather than any single one, when applying these techniques in the classification of hematologic neoplastic disorders.

2. *Diagnosis criteria*

All the disorders described here have been classified according to the criteria recommended by the W.H.O. classification of leukemias and hematosarcomas [30], and all the material has been studied in patients in an active phase of their disease.

Pathological cells from the peripheral bloods and/or the bone marrow of 132 patients with leukemic syndrome or dysglobulinemia have been investigated. These cases include: chronic lymphoid leukemia (CLL): 45 cases, typical primary acute lymphoid leukemia (typical primary ALL): 30 cases; typical acute myeloid leukemia (AML): 19 cases; leukemic lymphosarcoma (LLS): 15 cases; blastic crisis of chronic myeloid leukemia (CML): 6 cases; primary immunoblastic acute lymphoid leukemia (primary IALL) [31]: 4 cases; hairy cell leukemia (HCL): 4 cases; myeloma: 3 cases; primary macroglobulinemia: 2 cases; acute monocytoid leukemia (AMoL): 2 cases; chronic proplasmocytic leukemia (with macroglobulinemia) (CPL): 1 case; [32] chronic monocytoid leukemia (CMOL): 1 case.

In addition, pathological cells from lymph node biopsies of 18 patients with non Hodgkin's hematosarcomas, including 16 non leukemic lymphosarcomas (non leukemic LS) and two reticulosarcomas (RS) have been studied.

RESULTS

1. *General classification*

Table 5 presents the four different membrane phenotypes which have been encountered in this study, when classifying diseases according to immunological markers: 10·6% of the cases studied (16/150) have been typed as T (Group I), 44% (67/150) as B (Group II), while 40% of cases (61/150) were found to have cells with no detectable membrane markers in the technical conditions used (Group III), and 4% (6/150) to have cell bearing activated complement and/or Fc receptors, which make these disorders difficult to identify (Group IV).

Table 5. *Four different membrane phenotypes in human neoplastic hematological disorders*

I	mIg (−), Fc ag Ig (−)[*],	C_3 (−), E (+), Em (−)	T
II	mIg (+), Fc ag Ig (+),	C_3 (+), E (−), Em (+)	B
III	mIg (−), Fc ag Ig (−),	C_3 (−), E (−), Em (−)	NDMM
IV	mIg (−), Fc ag Ig (+),	C_3 (+), E (−), Em (−)	?

[*] may be (+) on certain T neoplastic cells.

For abbreviations, see Table 2.

2. *Analysis of the different disorders according to the four immunological groups*

2.1. *T cell neoplasia* (Group I). Table 6 summarizes our results: 50% of cases (8/16) of T cell disorders are primary ALL, while 50% of the remaining cases include five cases of LS,

Table 6. T cell neoplastic disorders

Observed membrane phenotype: mIg (−), Fc ag Ig (−), Fc (−), C_3 (−), E (+)		
1. Primary immunoblastic acute lymphoid leukemia	25%	1/4*
2. Typical primary acute lymphoid leukemia	23%	7/30
3. Non leukemic and leukemic lymphosarcomas	16%	5/31
4. Blastic crisis of chronic myeloid leukemia	16%	1/6
5. Chronic lymphoid leukemia	4%	2/45
Total	10·6%	16/150

*Fc ag Ig (+).

two cases of CLL and one case of blastic crisis of CML.

From this data, it is confirmed that in ALL, one case out of four is classified as T, while so called T cell CLL remains quite exceptional since we found only two such cases out of 45 typical CLL investigated for membrane markers.

2.2. *B cell neoplasia* (Group II). Table 7 summarizes our data. Most B cell proliferation were CLL, leukemic and non leukemic LS. Multiple myeloma and primary macroglobulinemia were, as expected, also of B cell type.

All these disorders have been shown to be monoclonal, most often of the IgM type, since the same immunoglobulin has been detected on the majority of neoplastic cells. In CLL (Table 8) the membrane IgM was often found to be associated with an IgD of the same light chain, while this association was less frequent in the case of leukemic and non leukemic LS [33].

2.3. *Neoplastic disorders with no detectable membrane markers* (Group III). As presented in Table 9, this category of disease includes all cases of AML (19/19) and all cases of RS (2/2) which were studied as well as a majority of cases of primary typical ALL (23/30) and of blastic crisis of CML (5/6). Note that in 26% of cases of non leukemic and leukemic LS (8/31) there were no detectable T and B membrane

Table 7. B cell neoplastic disorders

Observed membrane phenotype: mIg (+), Fc (+), C'3 (+), E (−)		
1. Myeloma (Kahler)	100%	3/3*
2. Primary macroglobulinemia (Waldenstrom)	100%	2/2
3. Chronic proplasmocytic leukemia (with macroglobulinemia)		1/1
4. Chronic lymphoid leukemia	88%	40/45
5. Non leukemic and leukemic lymphosarcomas	58%	18/31
6. Hairy cell leukemia	50%	2/4
7. Primary immunoblastic acute lymphoid leukemia	25%	1/4
Total	44%	67/150

*Positive intracytoplasmic immunofluorescence when using fixed smear.

Table 8. Immunological heterogeneity in chronic lymphoid leukemia (CLL)

I:	40/45 cases: B CLL because of prevalence of mIg (+) cells.
II:	36/40 cases monoclonal IgM: 29, IgG: 6, IgA: 1, mixed pattern: 4.
III:	All mIg (+) cells do not possess other receptors mIg (+), Fc ag Ig (+), C_3 (+), Em (+). 85% 30% 40% 51%
IV:	Monoclonal Ig D with the same light chain are detected on 15 to 80% of IgM (+) cells.
V:	5/45 cases: CLL without B membrane markers 2 cases: T CLL because of prevalence of E rosettes forming cells 3 cases: Unclassified CLL because of absence of detectable T and B cell membrane markers.

Table 9. *Neoplastic disorders with no detectable membrane markers*

Observed membrane phenotype: mIg (−), Fc ag Ig (−), C_3 (−), E (−)		
1. Acute myeloid leukemia	100%	19/19*
2. Reticulosarcomas	100%	2/2
3. Blastic crisis of chronic myeloid leukemia	83%	5/5
4. Primary acute lymphoid leukemia	76%	23/30
5. Primary immunoblastic acute lymphoid leukemia	50%	2/4
6. Acute monocytoid leukemia	50%	1/2
7. Leukemic and non leukemic lymphosarcomas	26%	8/31
8. Chronic lymphoid leukemia	2%	1/45†
Total	40%	61/150

*Including acute myelomonocytoid leukemia.
†RFC with murine RBC.

markers. A similar result was found in one case of CLL.

2.4. *Neoplastic disorders with complement and/or Fc receptors* (Group IV). Results dealing with this group are presented in Table 10. There are two cases of HCL, two cases of CLL, one case of AMoL and one case of CMoL.

DISCUSSION

From the data reported here, hematological neoplastic disorders have been classified into three main groups, the T and B cell neoplasias and a third group of null cell disorders characterized by the proliferation of neoplastic cells having no detectable membrane markers T or B.

This immunological classification led us to several conclusions.

1. B cell neoplasias are the most frequent diseases in our series, since they represent about 50% of the cases studied.

All the B cell proliferations studied for monoclonicity have been found to be monoclonal, confirming other reports [9, 34, 35].

In our series, the monoclonal Ig is most often of the IgM type, and this monoclonal IgM is found to be associated in several cases studied with an IgD having the same light chain, also confirming previous reports [36–38].

Except for some cases including immunoblastic acute lymphoid leukemias [31] and lymphosarcomas [19, 33], all the disorders of this group have been found to possess various common characteristics. They occur in adults, they have a chronic or subacute evolution, and the neoplastic cells appear relatively well differentiated. These disorders however, may represent different neoplastic processes. There is a temptation to assume, as do Salmon and Seligman [17] that these various proliferations may be due to the blocking of the B cell maturation, at different levels, but this concept needs further investigation.

2. In contrast, T cell neoplasias are less frequent since this group represents only about 10% of our series. Except for two cases of CLL, this group is composed of acute diseases including leukemias and lymphosarcomas. It is worth mentioning that most of the patients in this group are children or young adults [16, 33, 34].

Table 10. *Neoplastic disorders with activated complement and/or Fc receptors*

I—Observed membrane phenotype: mIg (−), Fc ag Ig (+), C_3 (+), E (−)		
Hairy cell leukemia	50%	2/4
Chronic lymphoid leukemia	4%	2/45
II—Observed membrane phenotype: mIg (−), Fc ag Ig (−), C_3 (+), E (−)		
Acute monocytoid leukemia	50%	1/2
Chronic monocytoid leukemia		1/1
Total	4%	6/150

These observations suggest that T cell diseases may be related to thymic anomalies since they affect young patients whose thymus should normally be functional and since experimental data exist demonstrating the role of the thymus in animal leukemogenesis [4, 12, 15, 16]. This hypothesis needs further experimental confirmation.

3. In our series, 40% of the cases have not been classified as T or B cell diseases, and thus have been called "null" cell disorders. Except for one case of CLL, this group was found to be composed of a majority of the cases of acute leukemias and of 26% of the non-Hodgkin hematosarcomas studied. These data led us to hypothesize that this group of null cell disorders may, in fact, cover at least two types of diseases.

The first type includes non-lymphoid neoplastic proliferation, i.e. diseases of myeloid or monocytoid origin, since all the AML cases investigated for T and B membrane markers were included in this group. In contrast, the second type may be composed of true lymphoid disorders in which neoplastic cells are characterized by a lack (or a loss) of T or B membrane markers, probably due to modifications induced through the neoplastic process: this hypothesis could explain the presence of several cases of undifferentiated leukemias and non-Hodgkin's hematosarcomas in this group.

The demonstration of the individualisation of null cell diseases, however, raises the question of the existence of undifferentiated diseases not related either to the lymphoid or to the myelomonocytoid series. These may be disorders of the stem cells (Davies, 1975).

It is evident that more sophisticated investigations including the use of cytochemistry [39, 40] and of specific hetero-antisera [21, 41] will be helpful to elucidate this problem.

4. We described a fourth group of neoplasias which includes a limited number of cases (4% of our series). In this group the neoplastic cells possess activated receptors for complement and/or Fc receptors and thus could not be definitely typed as B lymphocytes or monocytes in nature. It is believed that these disorders may, in fact, have a B lymphoid or monocytoid origin and that the lack (or the loss) of the other markers of the B or monocytoid series may be due to the occurrence of a time related functional variation of the synthesis of these receptors.

From these data it is obvious that it is through an extended use of such immunological investigations that progress will be made in the improvement of lympho-monocytoid proliferative disorders. However, definite progress cannot be achieved if immunological data are not correlated with other parameters such as the clinical picture, the prognosis factors as well as the results of cytology, histology and other conventional techniques. The search for such a correlation between clinical and morphological data will be the subject of other papers [19, 33, 42,].

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Increase in E. Active Rosette Forming Lymphocytes in Melanoma Patients Treated with BCG*

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Abstract—*E active rosette forming lymphocytes, a subpopulation of T lymphocytes, were studied in 14 tumor free melanoma patients undergoing BCG immunostimulation. In 7 patients, low values of Ea rosettes were observed before or at the beginning of the treatment, while total T lymphocytes were found to be within the normal range. In these patients, after BCG treatment was instored Ea rosettes increased and reached the normal range, suggesting that BCG could act on T lymphocytes.*

INTRODUCTION

SEVERAL authors [1–3] have described T cell deficiency in cancer patients at various stages of the disease. Recently, Wybran *et al.* [4] have introduced a new marker for a sub-population of T lymphocytes: the Active Rosette test which detects a sub-population of T lymphocytes which may be decreased in some pathologic circumstances including cancer. We studied this test in melanoma patients submitted to BCG immunostimulation.

MATERIAL AND METHODS

Patients

Fourteen melanoma patients were studied. All of them were bearing localised melanoma, histologically confirmed and classified as Clark's grades 3 and 4. After surgical removing of the tumor, they were submitted to BCG treatment according to the E.O.R.T.C. protocol: 6×10^8 living bacteria from Pasteur Institute were administered intradermally, using the Heaf Gun multiple puncture apparatus Eschman's Mark [5]. Stimulation was performed weekly, then once a month according to the skin reaction of the patient.

Laboratory investigation

Venous blood samples were collected on calciparin and lymphocytes were isolated by centrifugation on Ficoll Radio selectan Gradient [6].

Total rosette forming lymphocytes were appreciated using a slight modification of the technique described by Jondal [7].

Active rosette forming lymphocytes were determined according to Wybran [4]. Immunoglobulin bearing lymphocytes as detected by immunofluorescence using a goat anti-immunoglobulin conjugate (Meloy Laboratories, Inc. Springfield, Va, U.S.A.) were considered as B lymphocytes.

RESULTS

The normal value of active rosette forming lymphocytes was found to be $28 \pm 4{\cdot}9\%$ as determined in 30 healthy donors. Three patients showed normal values of Ea rosettes before the beginning of BCG treatment and these values did not vary during the first five months of the treatment.

Seven patients were demonstrated to have low values (from 10·8 to 25%) before or at the beginning of BCG treatment. These values increased during BCG treatment, more or less rapidly according to the patient, then stabilized within the normal range (Fig. 1).

Four patients entered the study after the 6th or the 12th month of BCG stimulation. They

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presented normal values of Ea rosettes and these values were stable within the normal range for the 5 subsequent months.

In none of these patients, B and total T lymphocytes could be demonstrated to vary out of the normal range.

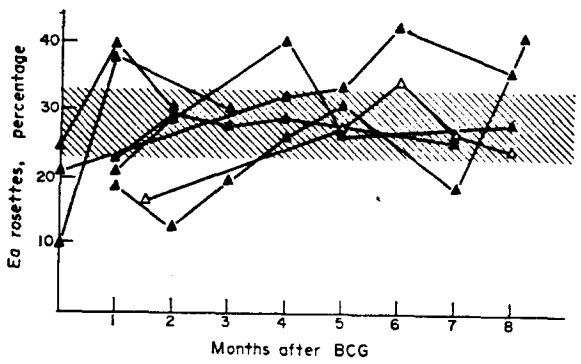

Fig. 1. Variations of Ea rosettes percentage in melanoma patients treated by BCG—the hatched area corresponds to normal values.

DISCUSSION

Since attempts to stimulate immunity have been introduced in solid tumors treatment [8, 9] few studies have dealt with a biological test which could allow to follow up the treated patients [10–12].

Using Active rosette test we were able to detect T cell deficiency in 7/10 melanoma patients, at the localised stage of their disease and who presented at that time normal values of total T lymphocytes as determined by total red cell rosetting.

Furthermore in these patients BCG treatment seemed to induce a restoration of this subpopulation and showed no biological effect in patients having normal values of Ea rosettes. Parts of these results are in agreement with those of Claudy *et al.* [13]; however, these authors did not detect any increase of Ea rosettes under BCG, but there were differences in the dose and mode of BCG administration.

It has been assumed that T lymphocytes with high affinity receptors for sheep red cells detected by Active rosette test are actively engaged in cellular immunity [4], and it is possible to make the hypothesis that BCG could act through activation or induction of T cell differentiation. However, there is very few information on the precise function of this T lymphocyte subpopulation [14] and it cannot be excluded that increase in Ea rosettes is only a sign of BCG effect, but without relation with its precise mechanism of action in immunotherapy.

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Serum Migration Inhibitory Activity in Patients with Infectious Diseases and Various Neoplasia*

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Abstract—*The presence of leucocyte migration inhibition activity was studied in the sera of healthy donors and of patients with tuberculosis, infectious mononucleosis and various cancers, namely carcinoma of the bronchus and breast and glioblastoma. Normal subjects do not exhibit any LIF activity in their sera. In contrast, 39 of 50 patients with tuberculosis and 4 of 5 patients with mononucleosis showed a positive reaction.*

In the cancer group, LIF activity was detected in 52 of 99 cases of bronchus carcinoma, 13 of 49 with breast carcinoma and 21 of 45 with glioblastoma. These results were obtained by using an improved technique which detects the presence of migration inhibitory factors produced in vivo in the serum and suggest that a persistent antigenic stimulation is required for the production of such an inhibitory activity.

INTRODUCTION

METHODS for detection of migration inhibitory activity in the serum are commonly used as parameters of delayed hypersensitivity [1]. Numerous techniques have been developed using human peripheral leucocytes as indicators cells and considerable variation in the results has been observed [2, 3].

In a number of studies, it was possible to detect a migration inhibitory factor (MIF) in the serum of animals with delayed type hypersensitivity reactions [4–6]. Moreover the presence of MIF or MIF-like activity has been reported in the serum of patients with various lymphoproliferative diseases, Hodgkin disease, chronic lymphocytic leukemia, myeloma [7] and Sezary syndrome [8], in patients with post transplantation hepatic dysfunction [9] and in the joint fluid of patients with rheumatoid arthritis.

In this study MIF activity was detected in the sera of patients suffering from infectious diseases, namely tuberculosis and mononucleosis, and three types of cancer disease, bronchus and mammary carcinoma and glioblastoma. The technique, originally described by Beaulieu, employs unidirectional migration of leucocytes in microhematocrit capillaries.

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MATERIAL AND METHODS

Patients

Twenty-four normal subjects ranging in the same age as the patients served as controls. Of a total of 247 patients studied; 50 suffered from tuberculosis confirmed by X-rays and cultures, in another 5 the diagnosis of infectious mononucleosis was established on the presence of atypical lymphocytes in the blood smear with a normal bone marrow and the presence of heterophil antibody in the serum.

The cancer patients were selected as follows: 99 cases of non-resectable squamous cell bronchial carcinoma, 95 cases of post-resectional recurrent relapse of glioblastoma multiformis and 49 cases of metastatic or disseminated breast carcinoma.

Sera

Ten ml of blood were collected from each patient and from 24 healthy volunteers. The blood was centrifuged at 800 *g* for 10 min and the sera stored at 4°C until testing within two-weeks' time.

Leucocyte migration inhibition (LMI) assay

An original technique, developed by Beaulieu [10], was used in this work. 20 ml of whole blood was drawn from a normal donor and was allowed to settle with 6% dextran in saline into a plastic syringe containing sodium citrate as an anticoagulant. After sedimentation of red

blood cells, the plasma layer was centrifuged and the white blood cells pellet was incubated for 7 min at 37°C in a 0·83% solution of ammonium chloride to lyse erythrocytes. The cell suspension was then washed and adjusted to 4×10^7 cells per ml in RPMI 1640 culture medium supplemented with antibiotics. This suspension was aspirated under negative pressure into disposable polystyrene tubes PP20 plugged at one end by parafine wax. The capillaries were spun for 3 min at 500 *g*, cut with a blade at the cell-fluid interface and inserted into microhematocrit chambers filled with a medium containing 50% decomplemented serum of the patients to be tested and 50% RPMI 1640 culture medium. The hematocrit chamber was sealed, fixed on a glass slip with silicone grease and incubated for 18 hr at 37°C.

The migration inhibition index (MI) was calculated as follows:

$$MI = \frac{\text{Migration length in medium containing serum sample}}{\text{Migration length in control serum}}$$

Two independent assays, each consisting of two capillary tube preparations were performed for each serum. Inhibition of 20% or more was considered as significant.

RESULTS

This modified leucocyte migration inhibition assay was able to detect an inhibitory activity in the serum of patients suffering from the infectious diseases already mentioned, the results having been summarized in Table 1.

Table 1. Leucocyte migration inhibition (LMI) by sera of normal donors and patients with various diseases

	Positive LMI assay	Negative LMI assay	Total
Normal donors	1	23	24
Tuberculosis	29	21	50
Mononucleosis	4	1	5
Squamous cell bronchial carcinoma	52	47	99
Breast carcinoma	13	36	49
Glioblastoma multiformis	21	24	45

No inhibition could be detected in the sera of 24 healthy subjects with the exception of one. In contrast, inhibition was observed in 39 of 50 cases of tuberculosis (78%) and 4 of 5 patients with mononucleosis (80%).

In the cancer groups we obtained positive results in 52/99 cases of squamous cell bronchial carcinoma (53%), 13 of 49 patients with breast carcinoma (27%) and 21 of 45 cases of glioblastoma (46%). The variation of the migration index showed no differences among diseases, ranging from 0·42 to 1·12.

We obtained reproducibility between replicates, the error being almost always within 10%.

DISCUSSION

The microtechnique employed in this work for detecting LIF activity in the serum offers considerable advantage as allowing a large number of replicates for a small amount of blood as source of indicator cells and very small quantities of serum for testing. Besides, the unidirectional migration of the cells is very easy to read and is not affected by external factors. This contributes to a good reproducibility of the test and to a minimal variance between replicates.

Both mononuclear and polymorphonuclear leucocytes are present as migrating cells in the capillary tubes [11, 12]. The indirect migration assays already described [13] detect migration inhibitory factors (MIF, LIF, etc. . . .) produced *in vitro* by sensitized lymphocytes whereas in our study, we tested the inhibitory activity of such factors already produced *in vivo*.

Healthy controls did not usually yield an inhibitory activity in their sera in contrast to the patients with tuberculosis, mononucleosis and the various cancers. The results suggest that the stimulus required to produce inhibitory factors in amounts sufficient to allow their detection in serum could be a persistent stimulation by virus, bacilli and tumor antigens.

In this study, the nature of the factor(s) responsible for the inhibition of leucocyte migration is not elucidated and antigen-antibody complexes or immunoglobulins cannot be excluded in the populations [14, 15]. The lack of response in the populations studied especially in the cancer patients, suggests that an antigenic stimulation is not enough to cause LIF production. Immune competence of the host is required as there is a very close correlation between delayed hypersensitivity skin responses to various antigens and positive migration inhibition assays [12, 16–18]. We were able to confirm this correlation, the results appearing in our next paper.

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Correlation of Delayed Hypersensitivity and Leukocyte Migration Inhibition Factor in the Serum of Cancer Patients

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Abstract—*Cellular immunity was evaluated in patients with three types of cancers: squamous cell bronchial carcinoma, breast carcinoma and glioblastoma multiformis.*

Delayed hypersensitivity skin responses to DNCB as primary antigen and to PPD, candidin and mumps as recall antigens were measured in these patients. At the same time, the presence of leucocyte inhibition factor (LIF) in the serum was assayed.

A good correlation between the degree of inhibition of migration and the size of the skin reaction was found in patients with bronchus and breast carcinoma but not glioblastoma.

Following these patients up during the course of their disease, by the in vivo *and* in vitro *tests, a good evaluation of the immunological reactivity of the patients could be obtained.*

INTRODUCTION

CELL MEDIATED immunity (CMI) in patients with malignancies has been clinically evaluated *in vivo* by delayed skin reactivity to a battery of antigens injected intradermally. The use of dinitrochlorobenzene (DNCB) has recently offered an advantage over bacterial antigens since exposure to DNCB does not usually occur and initial application evokes a true primary sensitization [1, 2]. *In vitro* assays for testing cell mediated immunity include lymphocyte responsiveness to non-specific mitogens and tumor specific lymphocytotoxicity tests.

Many attempts to compare the results obtained from different *in vivo* and *in vitro* assays have often failed to yield significant correlations [3–5].

In a previous paper [6] we were able to detect, in the sera of patients, the presence of a leukocyte inhibitory activity which is considered as an *in vitro* reflection of CMI [7–9].

Our purpose in this study is to compare the results obtained by two assays: the elicitation of delayed cutaneous hypersensitivity to DNCB and recall antigens, and the presence of LIF in the serum of patients with squamous cell bronchial carcinoma, breast carcinoma and glioblastoma multiformis. We have, moreover, followed the variation of the results of these assays during the course of the diseases mentioned.

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MATERIAL AND METHODS

Cancer patients studied in this work are those already described in the previous paper [6]. They all received intermittent chemotherapy treatment but the tests were performed 30 days after the end of the cycle.

Skin test with DNCB

Patients were sensitized locally with 2000 μg of 2,4-dinitrochlorobenzene (DNCB), and were challenged with a dose of 200 μg, 14 days after

sensitization. The skin was examined 48 hr later. The reaction was considered positive if induration and erythema were present on at least the half of the sensitization area.

Skin tests with recall antigens

These included tuberculin 10 U, mumps antigen 2 U, and candidin 2 U. One tenth ml of each preparation was injected intradermally on the volar surface of the forearm. Skin tests were read at 36 hr and 48 hr, and were considered positive if induration in the erythema area exceeded 5 mm dia (in two of three reactions) [10].

Leukocyte migration inhibition assay (LMI)

This test is described in detail in the previous paper [6]. Statistical analysis was performed by chi square test (χ^2) using Yates correction.

RESULTS

Impaired reactivity to DNCB and to recall antigens was observed in 28/42 and 37/99 respectively for bronchial carcinoma patients, in 9/10 and 39/49 for breast carcinoma patients and in 13/22 and 20/45 for glioblastoma patients (Table 1). The number of patients tested with DNCB is lower because the two-weeks' interval needed for sensitization and challenge was considered to delay treatment too much. Statistical analysis showed that there existed a significant correlation ($P < 0{\cdot}001$) between the LMI assay and the skin tests, both DNCB and recall antigens, for the patients with bronchus and breast carcinoma (Table 1). However, as far as the glioblastoma patients were concerned no correlation between skin tests and LMI assay was found. It appears that LMI assay correlates better with the response to DNCB but the number of patients tested is rather low to confirm this.

In the bronchus carcinoma group which included non-operable patients, it was more frequent to find negative LMI assay and positive skin tests rather than the opposite. Furthermore, the mean peripheral lymphocyte count was significantly lower in patients with negative LMI assays and negative skin tests.

In the breast carcinoma group, including stage III and IV patients, both LMI assays and skin tests were more frequently found to be negative.

We performed LMI assays and skin tests serially in three patients of each group during the course of their disease. In the case of bronchial carcinoma, time 0 corresponded to the pre-chemotherapy period. Several comments could be made on the results shown on Table 2.

In both bronchus and breast carcinoma, there exists a good correlation between skin tests and LMI assays, both showing the same variation during the evolution of the disease. This, however, is not true for the glioblastoma patients. In the terminal stages, both tests become negative, the LMI being the first. Fluctuations of the tests including conversion from an initial anergy to a positive status, and the opposite were observed to correlate well with remission and clinical improvement, or extension of the disease respectively. Finally, the size of the dermal reaction seems to correlate well to the degree of LMI.

DISCUSSION

This study shows that a significant depression of delayed cutaneous hypersensitivity could be observed in a variable proportion of patients particularly in patients with breast carcinoma (78%) who were all in an advanced stage of their disease. Correlation between anergy, as evaluated by skin tests, and absence of leukocyte migration inhibitory activity in the sera of patients with bronchus and breast carcinoma has been established in this study, suggesting that the detection of leukocyte migration inhibitory factor(s) (LIF) provides a meaningful *in vitro* correlate of cell-mediated immunity. The use of various antigens for skin tests, instead of one, is necessary to demonstrate this relationship. The reasons underlying the lack of correlation in glioblastoma patients could be the special localisation and histology of the tumour.

Other assays have been described and widely used for the evaluation of cell mediated immunity namely mitogen induced lymphocyte transformation, mixed lymphocyte culture (MLC), cell-mediated lymphocytotoxicity (CML) and the detection of lymphokine activity, results varying from significant correlation with delayed-type skin hypersensitivity to discrepancy [5, 11–15].

The good correlation found in this study may be due to the fact that skin test and LIF production are both dependent on sensitized lymphocytes capable of releasing soluble mediators of the cellular immunity.

Moreover, delayed hypersensitivity skin reactions are known to correlate well with the clinical course of the disease. Since the disappearance of LIF in the serum preceeds skin anergy, this LMI assay may be useful as an

in vitro test for prognosis. In conclusion, the LIF production requires both immunological competence and antigenic stimulation, the performance of the two tests allowing a good evaluation of the alteration of the immune status.

Table 1. Correlation between leucocyte migration inhition (LMI) assay, response to skin tests with primary and recall antigens and peripheral lymphocyte count (PLC)

Disease	*In vitro* test / *In vivo* test		Skin test with recall antigens +	Skin test with recall antigens −	Skin test with primary antigens (DNCB) +	Skin test with primary antigens (DNCB) −	P.L.C.
Squamous cell bronchial carcinoma	LMI	+	48	4	14	3	m = 1320 range: 370–3400
	LMI	−	14	33	0	25	m = 812 range: 150–3000
	Total		62	37	14	28	
Breast cancer	LMI	+	10	3	1	1	m = 804 range: 110–2500
	LMI	−	0	36	0	8	m = 632 range: 110–2500
	Total		10	39	1	9	
Glioblastoma Multiformis	LMI	+	15	6	6	4	m = 1418 range: 726–2610
	LMI	−	10	14	3	9	m = 1586 range: 500
	Total		25	20	9	13	

Table 2. Correlation between response to skin tests and presence of LIF in the serum of patients during the time course of their disease

Type of Disease	Number of weeks 0		8		16		20		28	
Squamous cell bronchial carcinoma	LIF	0%	LIF	52%	LIF	10%	LIF	10%	LIF	0%
	SKT	−	SKT	+	SKT	+	SKT	−	SKT	−
	LIF	0%	LIF	0%	LIF	0%				
	SKT	+	SKT	+	SKT	−				
	LIF	0%	LIF	60%	LIF	10%	LIF	0%		
	SKT	−	SKT	++	SKT	+	SKT	−		
Breast carcinoma	LIF	20%	LIF	0%	LIF	18%	LIF	33%	LIF	0%
	SKT	+	SKT	−	SKT	+	SKT	+	SKT	−
	LIF	0%	LIF	0%	LIF	0%				
	SKT	−	SKT	−	SKT	−				
	LIF	0%	LIF	0%	LIF	0%				
	SKT	+	SKT	−	SKT	−				
Glioblastoma multiformis	LIF	20%	LIF	0%	LIF	0%	LIF	0%	LIF	0%
	SKT	−	SKT	−	SKT	+	SKT	+	SKT	+
	LIF	0%	LIF	0%	LIF	+				
	SKT	+	SKT	−	SKT	−				
	LIF	25%	LIF	30%	LIF	+				
	SKT	+	SKT	+	SKT	+				

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Isolation and Electrophoretic Mobility of Three Lymphoid Populations in Normal Human Blood

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Abstract—*Cell electrophoresis allows separation of normal human blood lymphocytes into two main groups which are a function of their relative rates of migration, with regard to the reference speed* (1 $\mu m.sec^{-1}.V^{-1}.cm$) *: the lymphocytes which have a greater mobility than this value seem to be T-lymphocytes* (80·1 *per cent for* 42 *healthy adults*) *: on the contrary, B-lymphocytes have an inferior mobility* (19·9 *per cent*).

Two known methods are used for the selection of the lymphoid populations : spontaneous rosetting with sheep's red blood cells, which is characteristic of T lymphocytes, and adherence to nylon wool columns, which is dominant in the case of B-lymphocytes. This method confirms the fact that T-lymphocytes have a rapid migration and that B-lymphocytes a slow migration. We have isolated a third population, having neither the T markers nor the B markers. It has a very homogeneous migration, centered on the two classes 1·05 *and* 1·10 $\mu m.sec^{-1}.V^{-1}.cm$.

Lymphocyte electrophoresis, either analytical or preparative, allows the separation of cell populations, according to their rates of migration in an electric field [1].

We have carried out analytical electrophoresis on circulating lymphocytes with the same apparatus as described by Bangham *et al.* [2]. Generally two main kinds of lymphoid cells can be found in human blood: slow- and fast-moving lymphocytes. The electrophoretic mobility (E.M.) is less than 1 $\mu m.sec^{-1}.V^{-1}.cm$ for the slow-moving, and greater than 1 $\mu m.sec^{-1}.V^{-1}.cm$ for the fast-moving class. These two groups of cells can usually be subdivided into smaller, distinct sub-groups.

Several arguments allow us to believe that the rapid lymphocytes are the T-lymphocytes, and the slow-moving cells, the B-lymphocytes. These results have been obtained almost exclusively from animal experiments, in general with mice [1–5].

In man, there are only a few corresponding results [6–8]. In a group of 42 normal adults, we found approximately 20% of slow-moving lymphocytes; these results are similar to those of other authors [7], and approximate known figures on the distribution of normal B and T populations.

In an earlier work [9], we used methods known to enrich lymphoid populations: the separation of lymphocytes that do not form E-rosettes with SRBC, and the separation of cells adherent and non-adherent to nylon wool columns. This work showed the usual predominance of slow-moving lymphocytes among cells adhering to nylon wool and non E-RFC (rosette forming cells): while effluent lymphocytes on the columns have a rapid migration almost exclusively.

These results, correlated with immunofluorescence [10] seem to be supplementary arguments to affirm the T-nature of the fast-moving lymphocytes, and the B-nature of the slow-moving lymphocytes. In this work, the same methods of cell separation are used, in a combined utilisation which would allow us to obtain 3–4 different cell populations: for example, adherent or effluent lymphocytes, forming or non-forming E-rosettes.

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MATERIAL AND METHODS

The procedure, using 300 ml of normal human blood, has been described in detail elsewhere [10].

(a) *Lymphocyte separation* was performed by centrifugation on a Ficoll-metrizoate mixture (s.g. 1·077). The blood was diluted to 1/3 with 0·9% Na Cl, and centrifugated at 400 *g* for 30 mn [10].

(b) *Spontaneous rosetting with SRBC* was done in Erlenmeyer flasks according to a monothermic technique derived from that of Wybran *et al.* [11]. Each flask contained 2 ml of GIBCO foetal calf serum, 2 ml lymphocytes (10,000/mm^3) and 4 ml SRBC in Alsever (250,000/mm^3). After 3 hr at 25°C, or 16–18 hr at 4°C, and gentle manual resuspension, the cells were put through a second separation at 250 *g* on Ficoll–metrizoate [10]. In the second experimental series, E-RFC were collected on a new gradient after mechanical breaking up of E-rosettes, or after SRBC lysis with NH_4Cl, 0·87 percent.

(c) *Nylon wool column method* was very similar to that of Trizio and Cudkowicz [12], using 2 ml of the lymphocyte suspension (10–30,000/mm^3) per column [10].

(d) *Cell electrophoresis* technique uses the Rank Bros Mark 1 analytical electrophoresis apparatus [1] and has been fully described previously [6, 10].

(e) *Surface immunoglobulin staining* used Wellcome fluoresceinconjugated antisera [10].

RESULTS

The separation experiments were carried out on 11 healthy subjects, independent from the normal subjects used as a reference.

(a) *Electrophoresis on total lymphocyte blood population* allowed us to see a normal electrophoretic distribution, superposable to that of the control group (19·9 ± 5·6% of slow-moving, and 80·1 ± 5·6% of fast-moving lymphocytes in 42 normal, healthy adults whose average age was 46·5 years): (Tables 1 and 2).

(b) *First series: First E-Rosettes.* Five experiments were carried out with this protocol. The totality of the isolated lymphocytes was used to form E-rosettes with SRBC for 3 hr at 20–25°C. The percentage of E-rosettes were from 70, 69, 73, 70, 70% (the mean: 70%).

After a new separation on a Ficoll-metrizoate gradient, the cells which did not form E-rosettes were put on nylon wool columns. The RFC were recuperated by a mechanical breaking down, followed by a new gradient (see Methods).

Figure 1 shows that the three populations separated in this way have an E.M. that is quite different:

Population 1 corresponds to the ERFC, thus to an almost exclusive T population; its E.M. is found in the most rapid zone of rapid cells (centered around 1·25 μm.sec^{-1}.V^{-1}.cm). Table 1 shows that this population forms 86% E-rosettes, compared to 70% in the total population.

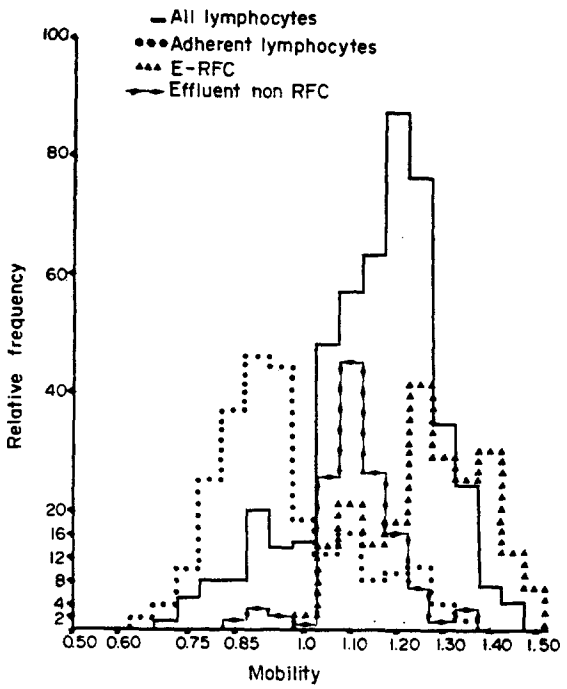

Fig. 1.

Population 2 corresponds to cells not forming E-rosettes, adhering to nylon wool columns. Thus, this population is, presumably, of a B-nature. Its E.M. is found in the slow-cell zone, centered around 0·86 μm.sec^{-1}.V^{-1}.cm. Table 1 shows 58% of these cells with a surface immunofluorescence (21% in the total population).

Population 3 is a population of cells that did not form E-rosettes, and that did not adhere to nylon wool. This population is characterized by a homogeneous E.M., limited to 2–3 classes of mobility, centered on 1·11 μm.sec^{-1}.V^{-1}.cm; and by less than 10% ERFC, or immunofluorescence-positive cells.

(c) *Second Series: First Nylon columns.* Six separation experiments were applied here. The totality of separated cells was placed into the nylon columns. Then, the two populations of adherent and effluent cells started forming

Table 1. *First Series: First E-Rosettes*

Cell populations	Total number of cells used ($\times 10^6$)	Per cent slow cells and mean E.M.		Per cent rapid cells and mean E.M.		Per cent E-Rosettes	Per cent Immuno-fluorescence positive cells
	250	23	0·860	77	1·172	70	16
	220	13	0·877	87	1·170	69	—
Total	470	14	0·880	86	1·167	73	25
lymphocytes	300	15	0·880	85	1·192	70	22
	96	21	0·923	79	1·165	70	—
	267	17·2	0·884	82·8	1·173	70	21
	95	5	0·835	95	1·242	80	
	21	0	—	100	1·285	87	
E-RFC	160	8	0·877	92	1·215	—	
	140	12	0·920	88	1·251	—	
	8			100		90	
	84	5	0·880	95	1·249	86	
	10	58	0·865	42	1·113	—	
Non E-RFC	6	67	0·892	33	1·090	—	
adherent	30	100	0·843	0	—	—	48
lymphocytes	20	73	0·048	27	1·072	—	68
	2	100	0·845	0	—	38	
	13·6	79	0·858	21	1·092		58
	2·4	5	0·850	95	1·135	10	
Non E-RFC	2	6	0·900	94	1·093	10	5
effluent	6	0	—	100	1·096	7	6
lymphocytes	4	3	0·950	97	1·110	8	5
	5·2	5	0·910	95	1·107	—	
	3·9	4	0·900	96	1·108	8·7	5·3

E-rosettes for 16–18 hr at 4°C. As before, the RFC and non RFC cells were recovered. A lesser number of the adhering cells that formed E-rosettes were recovered for electrophoresis on separated populations, compared to the total lymphocytes. (See Fig. 2).

1 *The total adherent population* had an E.M. that corresponds approximately to that of population 2 in the preceding series, and is found in the slow-cell zone. It contains a majority of B cells, and showed 60% of its cells to be surface Ig carriers. Nonetheless, it still contains ERFC (Table 2).

2. *The total effluent population* overlaps approximately the fast-moving cell zone. Its ERFC percentage is increased (80% compared to 68%). It contains only 5% surface Ig-carrier cells.

3. *The effluent RFC population* corresponds to population 1 in the preceding series. Composed almost exclusively of T-lymphocytes, it is characterized by a weak yield of RFC, due to a bad recovery on repeated gradients. The recovery was much better with the E-lysis with NH_4 Cl.

4. *The effluent non-RFC population* had an E.M. comparable to that of population 3 in the first series. As in this latter series, this population is characterized by a weak percentage of ERFC and of immunofluorescence-positive cells.

DISCUSSION

A large quantity of peripheral human lymphocytes form E-rosettes with SRBC under certain technical conditions [13, 14]. The ERFC are T-lymphocytes [15, 16]. In spite of a certain variability, it appears that the majority, if not all, of the circulating T-lymphocytes possess receptors for SRBC [17]. Thus, a great T-lymphocyte depletion could be established by eliminating ERFC. This is what we tried to do with a Ficoll-metrizoate gradient after a 3 hr (Series 1) or 16–18 hr rosetting (Series 2). This allows us to separate the cells that do not form E-rosettes. To obtain the RFC, the same

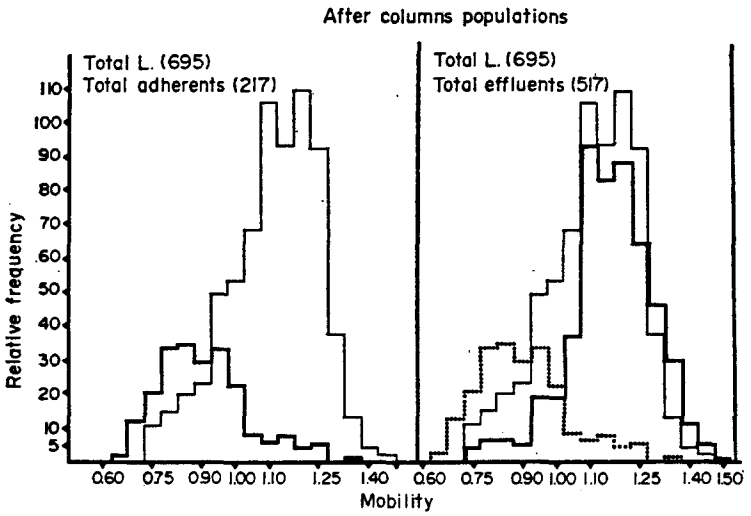

Fig. 2a.

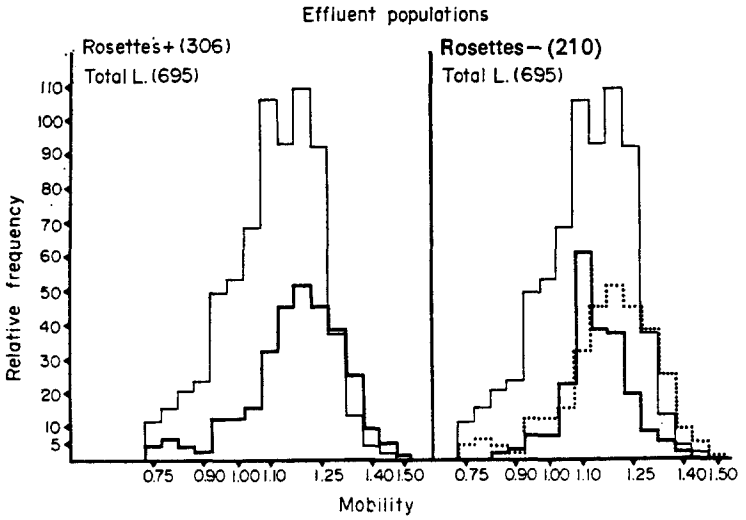

Fig. 2b.

procedure can be utilized again after a mechanical breaking down of the rosettes by manual agitation. But from our experience, the recuperation of RFC by this procedure is often tainted by a bad yield. The erythrocyte lysis by NH_4Cl was much more satisfactory for this purpose.

On the other hand, B-lymphocytes adhere to the nylon fibers more than the T-lymphocytes [17]. Indeed, the cells having a receptor for the complement [18], the precursors of antibody-forming cells [19], or the surface-immunoglobulin carriers [20] seem to adhere more to nylon or cotton wool that the lymphocytes not having these properties, which are characteristic of B-lymphocytes.

Our experimental results (Tables 1 and 2, Figures 1 and 2) show that combining the separation methods by spontaneous rosetting and nylon columns, allows three lymphocyte populations to be separated in normal human blood, whatever be the order in which these two methods are used. These 3 populations are characterized by their distinct electrophoretic mobility:

A population with a strong predominance of T (population 1 of Series 1, effluent E-RFC in Series 2) whose E.M. is centered around 1·20 $\mu m.sec^{-1}.V^{-1}.cm$.

A population having a strong predominance of B (population 2 of Series 1, the total adherent population of Series 2), whose E.M. is centered around 0·90–0·95 $\mu m.sec^{-1}.V^{-1}.cm$.

A third population with a homogeneous E.M., centered around the slow portion of the rapid lymphocyte zone, at 1·10 $\mu m.sec^{-1}.V^{-1}.cm$, neither adhering to nylon wool nor forming E-rosettes. The nature of this popula-

Table 2. Second Series: First columns

Cell populations	Total number of cells used or recovered ($\times 10^6$)	Per cent slow cells and mean E.M.		Per cent rapid cells and mean E.M.		Per cent E-Rosettes	Per cent Immuno-fluorescence positive cells
Total lymphocytes	600	16	0·895	84	1·168	71	25
	520	21	0·859	79	1·168	65	20
	300	21	0·875	79	1·172	70	20
	220	24	0·863	76	1·171	61	22
	320	24	0·865	76	1·181	70	
	326	18	0·876	82	1·164	74	
	381	21	0·872	79	1·171	68·5	22
Total adherent	30	67	0·845	33	1·081	33	60
	24	68	0·868	32	1·031	45	60
	30	73	0·865	27	1·115	8	40
	14	65	0·851	35	1·133	21	
	40					32	
	33					28	
	28·5	68	0·862	32	1·090	28	53
Total effluent	210	5	0·980	95	1·212	77	
	370	0	—	100	1·268	77	
	185	0	—	100	1·258	75	5
	350	10	0·880	90	1·275	89	
						81	
						71	
	279	4	0·930	36	1·253	78	
Effluent E-RFC	157	10	0·859	90	1·250	70	
	30	8	0·875	92	1·270	81	
	28	0	—	100	1·248	72	4
	6·5	0	—	100	1·198	59	5
	75	7	0·953	93	1·259	86	15
	70	6	0·950	94	1·228	90	
	61	8	0·909	94	1·242	76	8
Effluent non E-RFC	18	7	0·970	93	1·119	2	15
	2·4	0	—	100	1·122	13	
	14·4	8	0·920	92	1·108	14	
	11	5	0·960	95	1·129	14	10
	7·5					12	25
	7·5	8	0·963	92	1·153	18	5
	10·2	6	0·953	94	1·126	12	14
Adherent E-RFC		not done		not done		58	
						45	
						52	
	5·5					75	30
	3					85	35
						63	32·5
Adherent non E-RFC	3·5	not done		not done		0	
	4·5					3	50
						2·5	50
						2	50

tion can still only be supposed. Almost by definition, since it has neither T nor B-markers, it could correspond to what certain authors call "null" lymphocytes [22].

Several comments could be made on Figs. 1 and 2:

In the separated populations, a gain of very rapid cells is present when the population is predominantly rapid, and there is, equally, a gain of very slow cells in populations that are predominantly slow; the same phenomenon is responsible for the average mobility variations observed in the preceding work [10].

The rapid populations (T and "null" lymphocytes) do not "contaminate" the slow zone or only very weakly (generally, the limit is fixed at 1 $\mu m.sec^{-1}.V^{-1}.cm$ [1, 5, 7, 9, 10]. On the other hand, the B population seems to present a rather notable lag in the rapid moving cells' zone, up to about 1·20 $\mu m.sec^{-1}.V^{-1}.cm$.

The nylon wool method is sometimes criticized for its varying yields. Nonetheless, the yields obtained in our preceding work with nylon wool, were relatively regular and on the order of 63% [10]. Here, they are 75% in the second Series. It might be preferable to use the Sephadex G200 column method, covered with antiserum anti Fab [22]. Nonetheless, the recuperation of adherent cells with this latter method would seem to be rather difficult.

The separated populations here cannot be considered as really pure. Their average purity seems to be around 80%. This imperfect enrichment may be linked to the presence of a small fraction of T-lymphocytes adhering to the columns, and to a small fraction of B-lymphocytes that form E-rosettes. Our results are compatible with hypotheses put forth by other diverse authors. Yet they have shown an actual enrichment.

The loss of cells at each stage of the separation represents one of the major disadvantages of these procedures.

The main interest of this paper seems to us to lie in the fact that we found a lymphocyte population with a homogeneous E.M., and not presenting any T or B markers.

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Immunological Study of Axillary Lymph-nodes in Patients with Breast Cancer

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(With the technical help of M. E. TOUZE)

Abstract—*The comparative study of lymphocytes from peripheral blood and lymph-nodes was carried out by evaluation of the percentage of cells with surface Ig and of E and EAC rosettes in cancer patients and non-cancerous individuals.*

A significant difference could not be shown between the lymphocytes of lymph-nodes from breast cancer subjects and the lymph-nodes of individuals not having cancer, in the same age group.

On the other hand, if the breast cancer patients aged less than 61 *and those older than* 61 *are compared, a significant increase is observed in the number of cells with surface Ig and EAC rosettes, but also a significant reduction in the number of E rosettes.*

THE AIM of this was to study the immuno response at a local level since the exploration of the general response in different cancers usually does not show notable modifications except in lymphoproliferative illnesses.

MATERIAL AND METHODS

Patients with breast cancer

All patients were classed in stages II and III in the BLOOM classification and were aged from 35 to 85 yr. The lymph-nodes were taken after mammectomy in which the lymph-nodes were always shown to be free of metastatic invasion.

Controls

The controls were non-cancerous subjects aged less than 60, who underwent surgical intervention either for thyroid lobectomy or for hysterectomy.

Lymphocytes

The lymphocytes were isolated from peripheral blood on Ficoll gradient and from the lymph-nodes by teasing.

Methods

E rosettes were prepared following the technique of Wybran [1] with the following modifications: the erythrocyte/lymphocyte ratio was 40 and the reading was carried out after overnight conservation at 4°C.

The EAC rosettes were prepared after Bianco *et al.* [2]. The surface immunoglobulins (SIg) of the cells were studied by immunofluorescence according to Papamichail [3].

RESULTS

Comparative study of lymphocytes from peripheral blood and lymph-nodes

With respect to peripheral blood, a very large increase in the percentage of cells with surface Ig and EAC rosettes was observed in the lymph-nodes. On the other hand, the percentage of E rosettes was raised less.

The same variations as in the controls were observed in the lymph-nodes of patients with epithelioma of the breast.

If the patients with breast cancer aged less than 61 are compared with those older than 61, one observes, in the latter, a significant increase in the number of cells with surface Ig and the number of EAC-rosettes as well as a significant lowering in the number of E-rosettes.

If patients and controls of the same age group are compared (61 yr or less), no change is observed in the tests carried out.

DISCUSSION

The results obtained from the tests of E- and EAC-rosettes or from the cells with surface Ig,

Table 1. *Percentage of lymphocytes with SIg, EAC-R and SRBC-R in CONTROLS*

Markers	Peripheral blood		Lymph-nodes	
	No. tested	Mean ± S.E.	No. tested	Mean ± S.E.
SIg	7	14·40 ± 2·21	5	24·00 ± 1·26
EAC-R	10	11·80 ± 1·14	5	31·80 ± 3·39
SRBC-R	14	62·00 ± 2·15	6	48·33 ± 3·55

Table 2. *Percentage of lymphocytes with SIg, EAC-R and SRBC-R in patients with BREAST CANCER*

Markers	Peripheral blood		Axillary lymph-nodes	
	No. tested	Mean ± S.E.	No. tested	Mean ± S.E.
SIg	8	18·25 ± 2·45	18	29·88 ± 2·10
EAC-R	8	13·37 ± 1·55	17	37·58 ± 1·71
SRBC-R	7	45·75 ± 7·21	16	38·31 ± 3·05

Table 3. *Percentage of lymphocytes with SIg, EAC-R and SRBC-R in lymph-nodes*

Markers	A—Controls: age (Yr) < 61		B—Patients with breast cancer age (yr) < 61		C—Patients with breast cancer age (yr) > 61	
	No. tested	Mean ± S.E.	No. tested	Mean ± S.E.	No. tested	Mean ± S.E.
SIg	5	24·00 ± 1·26	11	(1)N.S. 23·09 ± 2·12	7	(2) P < 0·001 41·00 ± 3·03
EAC-R	5	31·80 ± 3·39	11	(1)N.S. 34·81 ± 1·74	6	(2) P ≠ 0·02 42·66 ± 2·75
SRBC-R	6	48·33 ± 3·55	10	(1)N.S. 45·20 ± 4·51	6	(2) P ≠ 0·02 28·66 ± 2·76

(1): N.S. for B compared to A.
(2): P for C compared to B.

show functional modifications in the lymphocytes of axillary lymph-nodes in breast cancer as a function of age of the patients, as has already been reported by Good [4]. Patients and controls appear identical, yet it must be stated that this is with patients free of nodal metastases and it is probable that the results would be different if one turned to individuals with metastases, as was shown by Fischer *et al.* [5] in studying the incorporation of tritiated thymidine [6].

Moreover, Ellis [6], by using the migration inhibition test (MIF), has shown that lymph-node lymphocytes were immunologically more reactive than those from peripheral blood and it will be necessary to use other techniques to advance our knowledge of the immune response at a local level.

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Immunoperoxidase Method of B Lymphocyte Recognition

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Abstract—*The use of antibody-enzyme conjugates allows the labeling of surface-immunoglobulin bearing lymphocytes. This technique gives similar results compared to the fluorescence of surface immunoglobulins. This is a mean of evaluating the ratio of B lymphocytes.*

The immunoperoxidase staining is effected on permanent slides, with fixed cells, and observed with a light microscope.

According to the way of incubation used, the results are shown to be different:

—by incubating antibodies labeled with peroxidase on fixed smears: 20% of normal cells are recognized. The labeling is pericellular and continuous.

—by incubating antibody with living cells, in suspension, 10–12% of the lymphocytes are recognized; labeling appears like caps on the cells. These results are discussed.

INTRODUCTION

IT HAS been shown that the use of antibody-enzymes conjugates allows the labeling of surface immunoglobulin-bearing lymphocytes [1].

Heterologous antiglobulin, labeled with horseradish peroxidase, according to the technique of Avraméas [1] incubated with separated human peripheral blood lymphocytes is used to know the rate of B cells.

The antibody is fixed on surface immunoglobulins during the immunological step of the reaction. An histoenzymatical reaction using diaminobenzidine shows the localisation of peroxidase and thus the place of antibody conjugate. A cytological staining with May–Grünwald–Giemsa shows obviously all the cells of the smear.

MATERIAL AND METHODS

During the systematic trials to set up this technique two ways were possible to obtain the labeling of B cells.

1. Lymphocytes are separated from venous blood by Ficoll [3]. Cells are washed three times with Hanks plus 5% fetal calf serum, to wash out all the human serum immunoglobulins. With the bottom, we effected on one first technique (on the left on Fig. 1) smears with the washed cells. Then these cells are lightly fixed, either with ethyl alcohol (90 parts) and formaldehyde (10 parts), or with methanol, for 5 min. The fixative reaction must be very light and always dehydrating. The antibody conjugate is incubated in wet chambers for 3 hr, at laboratory temperature. The smears are washed with saline, then dived in a solution of diaminobenzidine according to Graham and Karnovski [2]. Hundred ml of Tris–HCl buffer with a tip of spatula 33'diamino-benzidine tetrahydrochloride, filtered and added just before use with 2 drops of hydrogen peroxide 120 vol. Histoenzymatical reaction is

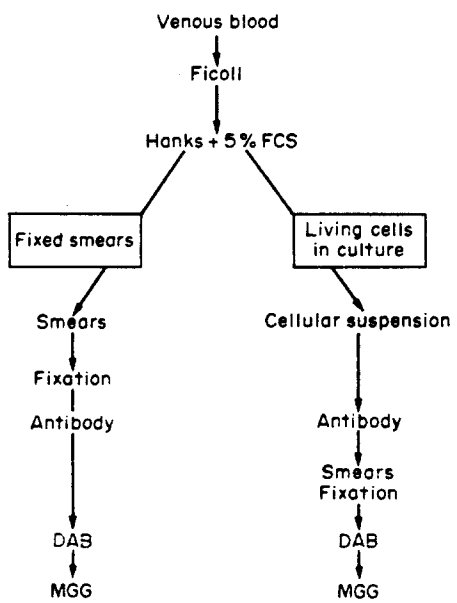

Fig. 1.

effected during 20 min. The smears are washed 3 times in saline, then coloured with Giemsa.

2. A second way (on the right of the Fig. 1) has been used:

The washed lymphocytes are incubated, living, in suspension in antibody for 3 hr, at 22°C. Then the cells are washed 3 times by spinning and resuspending the cells in Hanks and 5% FCS. Smears are effected, fixed, and the reactions with DAB and MGG staining follow the same way than for the first technique.

For each technique (on the left and right on Fig. 1), several smears, positive and controls are effected, as shown on Fig. 2.

Smears 5 and 6 include labeled cells and the count of B cell can be effected.

The most interesting control is the third one, done without antibody, because some cells have endogenous peroxidase that appears without any added enzyme.

Slide number	Interest
1 - Total heparinized blood	- % of lymphocytes
2 - Smears after Ficoll	- Cytological
3 - Without Ab. DAB MGG	- Endogenous peroxydase Polynuclears, Monocytes
4 - Ab. without DAB MGG	- Cytological
5 - Ab. DAB without MGG	- Labelled B Lymphocytes
6 - Ab. DAB MGG	- Labelled B Lymphocytes

Fig. 2.

RESULTS

We described two techniques, and some aspects of the cells are different. There are many things to note, seeing the smears:

1. Endogenous peroxidase is seen on smears 3, 5 and 6 (Fig. 2). The polynuclear cells, eosino on Fig. 3(a), neutro on 3(b), have endogenous peroxidase in lysosomes. The enzyme appears like big granules. These cells, easy to recognise are off count.

2. Reaction with DAB alone, without MGG (5 on Fig. 2) shows positive cells, but the negatives are very difficult to see. It is possible to look at these cells with phase contrast microscopy. Positive cells are surrounded with golden light.

3. With a total chain of reactions (Fig. 2, No. 6) B cells are labeled and it is easy to have the percentage of B cells (Fig. 3(c)).

4. During the two technical trials we saw that the labeling was morphologically different and the rate of the B cells was also quite different. As seen on Fig. 4, the first technique with previously fixed cells provides these results:
20% of normal donor cells are labeled. Labeling is pericellular, continuous, without capping, appearing on Fig. 3(d).

In the second technical way, by incubating living cells in suspension, cultured into antibody conjugates:
10–12% of cells are recognized.
Capping of surface immunoglobulins appears on the lymphoid cells (Fig. 3(e)).

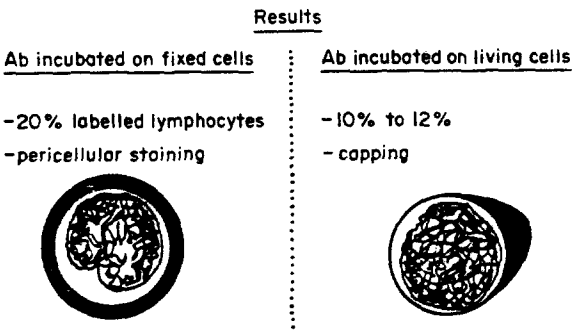

Fig. 4.

DISCUSSION

We think that the discussion of these last two results is interesting, as they appear to be different according to the technique used.

The fixative solution fixes the surface immunoglobulins. They cannot ride around the membrane when meeting a specific determinant [4]. This aspect of capping, observed by immunofluorescence [5] is here obtained according to the second technique in immunoperoxidase method. During the specific contact between surface immunoglobulins and the antiglobulins, the molecules of the cell membrane flock together at the top of the cell. This aspect has been observed by immuno-electron microscopy after incubating cells at 37°C with immunoperoxidase [6]. Capping of surface immunoglobulins appears in some lymphoproliferative diseases [7–8].

The second interesting result is the different rate of B cells obtained in the technical ways on Fig. 1.

We can presume that the good result is obtained by the second technique, by incubating the cells, cultured in antibody dilution. Some cells seem to bear immunoglobulins but cannot synthetise; they are labeled with the first technique, but not with the other. The

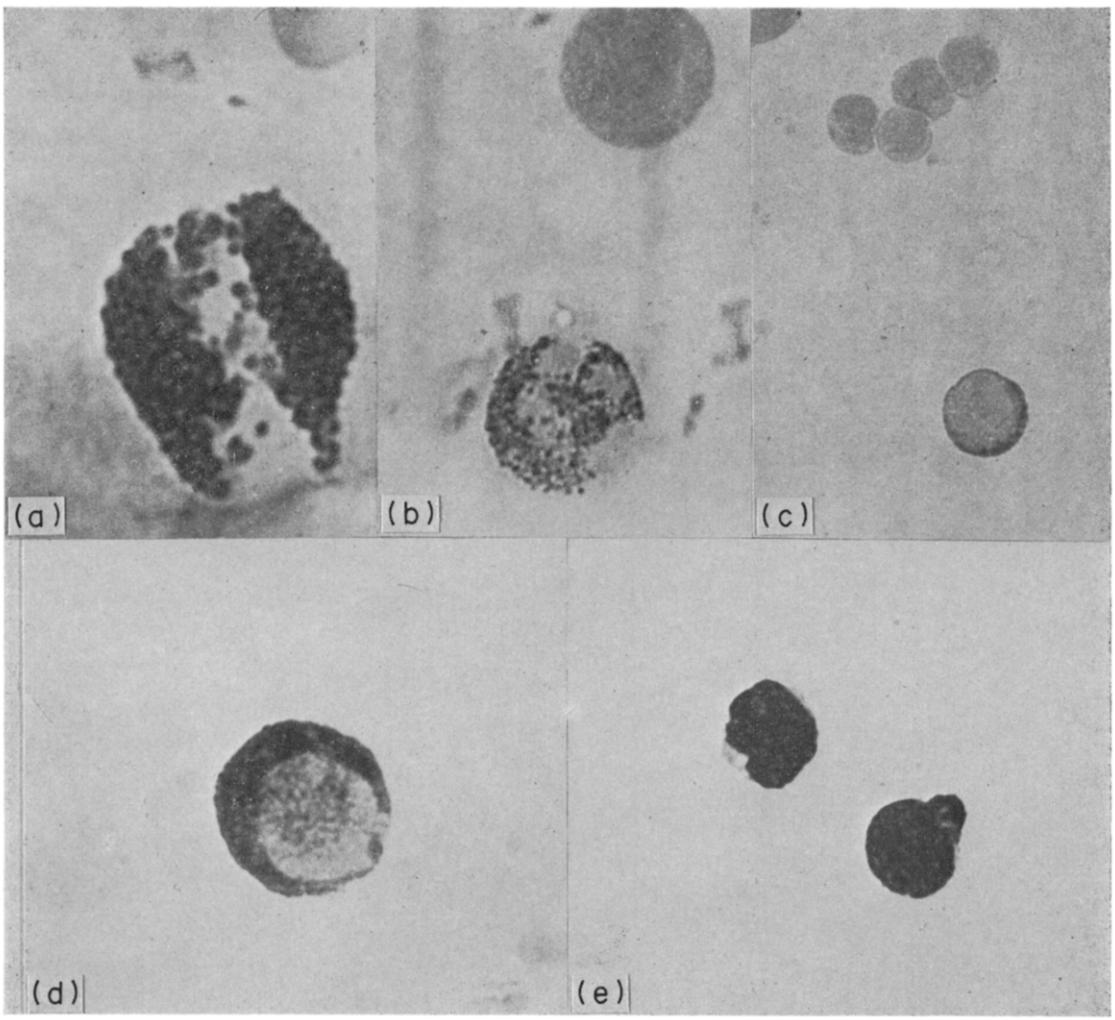

Fig. 3.

incubation during 3 hr with the antibody is equivalent to short term cultures. This seems to be very necessary before the counting of B-lymphocytes [9].

CONCLUSIONS

The immunoperoxidase recognition of B-lymphocytes provides similar results to those obtained with surface immunofluorescence. The immunoperoxidase method has yet been compared to surface immunofluorescence [10–11].

The advantages of this technique for lymphocyte staining include ease of preparation. No expensive material is needed; a light microscope is used to look at the cells. The cells are fixed and mounted on permanent slides. Many observers can see them, now or later on.

This technique is very close to the one used for immunoelectron microscopy [12–13].

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Monocyte Function in Cancer Patients

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Abstract—*Monocyte function has been studied in patients with lymphoma and lung cancer. Ability of monocytes to phagocytose Staphylococcus aureus was not depressed before treatment but was lowered by cytotoxic drugs. Recovery was then seen after a rest period. Bactericidal capacity was significantly lower in lymphoma patients prior to treatment than in normal controls. Chemotherapy lowered the bactericidal indices further and there was little recovery in between courses of treatment. Monocyte chemotaxis was depressed in untreated patients with advanced lung cancer and in half of a group of nineteen lymphoma patients. The implications of these monocyte defects are discussed.*

INTRODUCTION

MONOCYTE function is a credible requirement for an intact immune system. New roles in defence for the monocyte are projected almost weekly. Depressed monocyte function might be a predisposing factor in development of cancer. It might lead to inadequate containment of metastases and it might explain intercurrent infections frequent in tumour patients. As more aggressive treatment becomes the vogue, more patients develop serious immunodeficiency. It is no longer uncommon to witness resolution of a tumour such as a lymphoma followed by death due to overwhelming opportunist infection.

Peripheral blood monocytes have therefore been assessed for phagocytic and microbiocidal prowess in a group of lymphoma patients and appropriate controls before and after chemotherapy. A further group of lymphoma patients plus a small number of men with lung cancer have been tested for defects of monocyte chemotaxis.

MATERIAL AND METHODS

(1) *Cell preparation.* 20 mls of venous blood was collected into preservative-free heparin and separated on a Ficoll Hypaque column. The mononuclear cells collected from the gradient interface were washed, counted and adjusted to critical concentrations.

Phagocytosis and bactericidal capacity

Monolayers of 1×10^6 mononuclear cells were grown in 30% AB serum and Hepes solution for 22 hr. At that time 20×10^6 bacteria (Staphylococcus aureus) were added to the culture and the supernatants sampled 20 min later. Duplicate monolayers were then washed and lysed in distilled water at 0, 10, 20, and 40 min. The supernatants and lysates were subcultured on mannitol salt agar. From the bacterial counts at 48 hr the phagocytic capacity of the monolayer is calculated as a percentage of bacteria engulfed in 20 min of culture. The bactericidal index is an expression of the rate of killing of Staphylococcus aureus over 40 min.

Chemotaxis

A modification of Boyden's millipore technique was used. The chemo-attractant, either cobra venom factor (2·5%) or casein 5 and 10%, was placed in the bottom of a culture vessel and separated from 0·5 million monocytes in medium 199 by a millipore filter (pore size 5 μm). After incubation in a moist atmosphere at 37°C for 2 hr, the filters were removed, fixed in ethanol and stained with Giemsa solution. The distance that the "leading front" of monocytes had moved through the filter was measured in cultures containing medium 199 alone or medium 199 plus chemo-attractant. The first reading was subtracted from the second and expressed as the "distance migrated". In each experiment monocytes from a cancer patient were compared to those of an age, sex-matched control.

RESULTS

Phagocytosis

Eighteen patients were studied, each on several occasions. Before treatment patients' monocytes were as proficient as controls in phagocytosing Staphylococcus aureus. After one or more courses of combination chemotherapy (COP or MVPP)* phagocytosis was almost invariably inhibited. As shown in Fig. 1, five patients were studied serially: pre-treatment, on day 2 of chemotherapy and after two weeks clear of drug treatment. Depression of phagocytosis was seen while the patients were on treatment and some measure of recovery was seen prior to the next pulse of drugs.

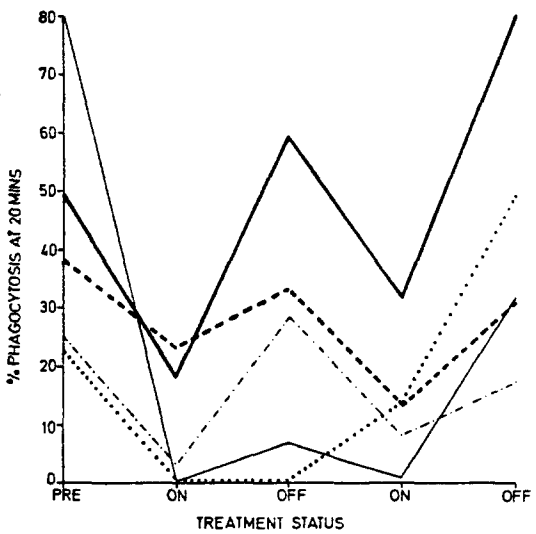

Fig. 1. Effect of cytotoxic drugs on monocyte phagocytosis.

Bactericidal index

Bactericidal capacity was estimated on over fifty occasions on eighteen patients. Their mean bactericidal index before treatment was 23 compared with the control value of 45. The difference was significant ($P < 0{\cdot}05$). The bactericidal indices of the study group fell gradually below pre-treatment values when drug treatment was initiated and did not recover between cycles. Indeed, in two patients the bactericidal index remained low three months after treatment had been discontinued and the patients' lymphomas had entirely remitted. Although monocyte and total white counts varied throughout treatment, fluctuations were shown to be independent of alterations in monocyte function.

Chemotaxis

A pilot study unrelated to the investigation of lymphoma patients above showed a marked chemotactic defect in monocytes from ten patients suffering from advanced lung cancer (Fig. 2). A group of nineteen lymphoma patients were therefore studied using the monocyte chemotaxis assay. None of the patients had received any treatment. The results were not as clear cut as those in the lung cancer group. Ten patients' monocytes were significantly less mobile than age, sex-matched controls; in seven patients the distances migrated by the study monocytes were similar to those of controls and two patients' monocytes were superior to the normals. No obvious correlation was noted between impaired chemotactic ability and histological type or stage of disease. The chemotaxis assays have not yet been repeated after treatment.

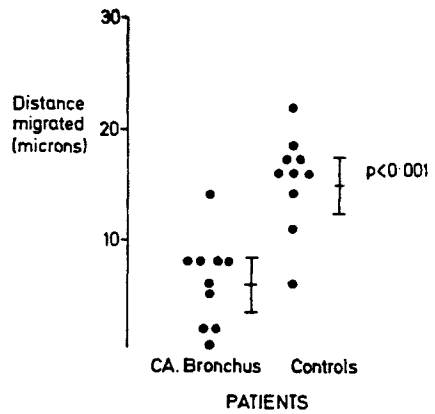

Fig. 2. Monocyte chemotaxis in carcinoma of the bronchus.

DISCUSSION

Impairment of monocyte function has been demonstrated in eight patients, with myelomonocytic leukaemia and two lymphoma patients [1]. Whether the deficiencies reported here represent an intrinsic cell defect or a secondary effect of a circulating factor is not yet elucidated. "Immunosuppressive factors" have been postulated in association with cancer, and in one group of patients [2] a small molecular weight polypeptide has been detected

*COP = cyclophosphamide vincristine prednisolone. MVPP = mustine vinblastine procarbazine prednisolone.

which inhibits phyto-haemagglutinin-induced DNA synthesis in lymphocytes. The latter factor was correlated with anergy, a notable accompaniment of lymphoma, particularly Hodgkin's disease. The drug-induced suppression of monocyte phagocytosis is predictable but would appear to be reversible in contrast to the progressive impairment of bactericidal capacity. It would be more pertinent to measure those functions with a gram negative organism such as Escherichia coli, as the infections incurred by the patients studied were usually of this type. The bacterial assays were, however, extremely unpredictable when gram negatives were employed so Staphylococcus aureus was retained as a standard.

Depression of monocyte chemotaxis is known to occur during viral illnesses such as influenza [3], again in association with loss of delayed cutaneous hyper-sensitivity and possibly a tendency to super-infection. It may be that tumours are not contained because of a defect in monocyte motility. Reversal of such a phenomenon is one of the aims of non-specific immunotherapy. BCG, C. parvum and levamisole may increase the pool of monocytes and conceivably increase their motility. It remains to be seen whether these measures have any anti-tumour effect or whether they may lessen the incidence of secondary immunodeficiency and opportunist infections in cancer patients.

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T and B Lymphocytes and Monocytes in the Spleen in Hodgkin's Disease: the increase in T Lymphocytes in Involved Spleens

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Abstract—*The percentages of T and B lymphocytes in the spleens of 40 patients suffering from Hodgkin's disease were studied. The results are summarized as follows: in involved spleens, i.e. containing Reed–Sternberg cells (RSC), there is significantly more T lymphocytes associated with significantly less B lymphocytes, in comparison to non-involved spleens. No significant difference was found between the two groups in the percentage of EA and EAC rosette-forming cells and non-T non-B mononuclear cells. There was no relationship between the histological type of the spleen and the percentage of either T or B lymphocytes.*

The demonstration of a higher percentage of T lymphocytes in 14 spleens containing detectable Reed–Sternberg cells (RSC) out of the 40 studied confirmed our preliminary data suggesting the existence of a local immunological reaction against Reed–Sternberg cells, in involved spleens in Hodgkin's disease.

INTRODUCTION

DURING the past few years several methods of investigation in man have permitted the identification of T and B lymphocytes [1–14] and of a third mononuclear cells population including monocytes [6, 15].

With different immunological techniques it is possible to characterize the mononuclear cells populations by different cell membrane markers (Table 1).

The purpose of this paper is to report our findings on the application of these techniques to the study of T and B lymphocytes and monocytes in the spleens of patients with Hodgkin's disease.

MATERIAL AND METHODS

1. *Patients*

The spleens of 40 patients with Hodgkin's disease (17 women and 23 men, aged from 11 to 61 years) were investigated.

Hodgkin's disease was diagnosed on clinical and histological criteria, and staging conformed to the Ann Arbor classification.

Before laparotomy, histological examination of pathological lymph nodes showed one patient to have lymphocyte predominance (LP), 21 to have nodular sclerosis (NS) and 11 to have mixed cellularity (MC). There were no cases of lymphocyte depletion (LD); 7 cases showed typical Reed–Sternberg cells (RSC) but could not be histologically subtyped for technical reasons (ND). Clinically, 2 patients were at stage I, 19 at stage II, 16 at stage III and 3 at stage IV.

After staging laparotomy, histological examination of the 14 spleens containing RSC (i.e. involved spleens) showed 6 NS varieties, 5 MC and 3 ND. Only 3 of the 21 patients at clinicals stages I and II had involved spleens whereas 11 out of 19 spleens were involved in the cases at disseminated stages III and IV ($0{\cdot}01 > P > 0{\cdot}001$).

Spleen weight was evaluated in each case. In our series, spleen weight was $309{\cdot}5 \pm 145$ (mean value) for involved spleens, and 176 ± 87

Table 1. Immunological test procedures used to differentiate T and B lymphocytes and monocytes

Immunological test	T lymphocytes	B lymphocytes	Third categories¶ Monocytes and histiocytes
E Rosettes* (SRBC receptors)	+	0	0
EA Rosettes† (Ig receptors)	(+)	(+)	(+)
EAC Rosettes‡ (Activated C'3 receptors)	0	(+)	(+)
Membrane Ig§	0	+	0

*Spontaneous rosettes with SRBC (see text).
†Rosettes with sensitized SRBC (see text).
‡Rosettes with SRBC antibody complement complexes (see text).
§Membrane immunoglobulins detected by direct immunofluorescence.
¶Non T non B mononuclear cells (see text).
(+)On some mononuclear cells.

(mean value) for non-involved spleens. This difference is significant ($P < 0{\cdot}001$).

2. *Mononuclear spleen cell purification*

The spleen fragments were gently disrupted and the cell suspension washed in Hank's medium. The mononuclear cells were isolated by lowering the suspension on a ficoll–triosil density gradient, according to a technique previously described [1–3].

The purity of the mononuclear cells was always checked by May–Grunwald–Giemsa staining and their viability determined by the trypan blue dye exclusion test.

3. *Immunological tests procedures for T and B cell quantification*

3.1. *T lymphocytes.* T lymphocytes were enumerated by the E rosette test using sheep red blood cells (SRBC) according to a technique already described [1–3].

Rosettes were defined as lymphocytes surrounded by at least 3 SRBC.

3.2. *B lymphocytes.* B lymphocytes were enumerated by determining the membrane immunoglobulins (mIg) by a direct immunofluorescent test using a polyvalent fluorescent isothiocyanate conjugated sheep anti-human immunoglobulin serum, as previously reported [1–3].

3.3 *Ig receptor (EA) and activated complement receptor (EAC) bearing mononuclear cells.* Enumeration of these mononuclear cell categories was performed by the EA and EAC rosette tests respectively. Details of these tests have also been reported elsewhere [1–3].

3.4. *Non-T non-B mononuclear cells (nT nBmc).* In addition, nT nBmc were calculated from the following formula: nT nBmc = mc − (T + B), in which mc = total number of mononuclear cells, T = total number of T lymphocytes, B = total number of B lymphocytes [16].

4. *Controls and statistical analysis*

Peripheral blood lymphocytes from 20 normal subjects were used as control and the results provided the normal range for each test.

Statistical analysis were performed using the Student *t*-test.

RESULTS

(a) *Immunological findings*

The results are summarized in Table 2.

In non-involved spleens (absence of detectable RSC), the mean percentage of E rosettes (T lymphocytes) was 35·5%. Extreme values ranged from 15 to 67%, with only 3 cases above 50%, whereas in involved spleens (presence of RSC), the mean percentage of mononuclear cells forming spontaneous E rosettes (T lymphocytes) was 50%, with extreme values ranging from 33 to 67% and only 3 cases below 42%. This difference is significant ($P < 0{\cdot}01$).

For EA and EAC rosettes, mean percentages were 16·5 and 22% respectively, for non-involved spleens. Extreme values ranged from 3 to 33% for EA rosettes and from 1 to 65% for EAC rosettes. In involved spleens, the mean percentages of EA and EAC rosettes were 17 and 14·5% respectively. Extreme values ranged from 1 to 51% for EA rosettes and from 1 to 35% for EAC. This difference is not significant.

In non-involved spleens, 31·5% of the mono-

Table 2. Membrane markers in mononuclear spleen cells (Hodgkin's disease)

Conditions	No.	B lymphocytes (membrane Ig) % Mean value	B lymphocytes (membrane Ig) % Extreme values	T lymphocytes (E rosettes) Mean value	T lymphocytes (E rosettes) Extreme values	EA and EAC binding mononuclear cells: EA Rosettes Mean value	EA Rosettes Extreme values	EAC Rosettes Mean value	EAC Rosettes Extreme values	Non-T non-B mononuclear cells % Mean value	Non-T non-B mononuclear cells % Extreme values
Non-involved Spleens	26	33·5* ± 13	13/70	33·5† ± 13·5	15/67	16·5 ± 12	3/33	22 ± 16	1/65	31(± 16)	0/65
Involved Spleens	14	23* ± 11	7/40	50† ± 11·5	33/67	17 ± 14	1/51	14·5 ± 12	1/35	27(± 13)	8/40

*$P < 0{\cdot}05$
†$P < 0{\cdot}01$.

nuclear cells (ranging from 13 to 70%) showed surface bound Ig (B lymphocytes). In involved spleens, the mean percentage of splenic lymphocytes bearing Ig on the cell surface was 23%, with extreme values ranging from 7 to 40%. This difference is significant ($P < 0{\cdot}05$).

Table 2 also shows the percentages of non-T non-B mononuclear cells (nT nBmc). In non-involved spleens the percentage was 31% (range: 0–63%) and in involved spleens 27% (range: 8–40%). No significant difference was detected.

(B) *Search for a correlation between immunological data and variables characterizing the disease*

This search led to three interesting observations:

(1) We found a significantly higher percentage of splenic T lymphocytes in disseminated clinical stages III and IV than in cases of localized disease (stages I and II). We also found a slightly fewer mIg binding mononuclear cells, (B lymphocytes) EAC rosette-forming cells and nT nBmc in the generalized disease. However, these differences were not significant. No variation was detected in the percentage of splenic EA rosette-forming cells in patients at different clinical stages of the disease.

(2) We compared involved spleens in the localized and disseminated stages and found no difference in the percentages of T lymphocytes. Nor was any variation detected when comparing T cells of the non-involved spleens in each clinical group.

(3) Finally, we found no clear-cut relationship in the spleen between the percentages of the different mononuclear cell populations and the histological type of the tissue.

DISCUSSION

The results presented in this paper confirm our previous findings [17–21] as well as those of others [22, 23] that in Hodgkin's disease there is a significantly higher percentage of T lymphocytes present in spleens containing RSC, than in non-involved spleens.

The mechanism of this increase is at present unclear. There are at least two possible explanations:

(1) Since it has been claimed that RSC may be T cell derived, this increase in T mononuclear cells may correspond to an increase in RSC precursors. This hypothesis does not seem plausible, however, because recent reports, including our own [18–21], have suggested that RSC may in fact, be derived from B cells [24, 25] or from histiocytes [26, 27].

(2) This increase in T cells may correspond to a local immune reaction against RSC. This would explain the strikingly close contact seen with conventional [28] and scanning electron microscopy [18–20] between presumed T lymphocytes and RSC. These morphological findings do not, however, prove the existence of a specific immunological reaction.

Our data regarding B lymphocytes confirm reports from other authors [29, 30] who found in using different methods a significant drop in the number of B cells in involved spleens. However, from our data we are unable to

deduce that there is a decrease in the absolute number of B lymphocytes in involved spleens in Hodgkin's disease.

The absence of any significant difference in the percentages of EA and EAC rosette-forming cells suggests that there is no difference in the percentage of cells related to the histiocytic series.

We showed a higher mean percentage of splenic E rosettes (T lymphocytes) in disseminated clinical stages III and IV than in localized stages I and II. According to our findings, there is no difference in the percentage of E rosette forming cells between involved spleens, whether they are localized or disseminated diseases. Our data showing a high mean percentage of splenic T lymphocytes in the generalized clinical stages may in fact be explained by a larger number of involved spleens in the disseminated cases. There is however, no clear correlation between the increase of T lymphocytes in the spleen and the degree of dissemination of the disease.

Our findings suggest that there is no correlation between the percentage of the different mononuclear cells and the histological type in the spleen; however, as our series was exclusively composed of NS and MC varieties, no definite conclusions can be drawn from these data.

Finally, from this study it is suggested that the detection of an increased number of T lymphocytes in involved spleens may be clinically helpful in the evaluation of the clinical stage of Hodgkin's disease when pathological analysis remains difficult to interpret.

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Differences in Mitogen Response and Electrophoretic Mobility of Two T-Lymphocyte Sub-Populations

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Abstract—*T-lymphocytes respectively respond to Phytohemagglutinin (PHA-P), to Concanavalin A (ConA) and Pokeweed Mitogen (PWM). The response to mitogens of T-lymphocyte sub-populations has been evaluated. These lymphocytes have been purified by carbonyl iron treatment and separation on Immunoabsorbant columns (anti-$(Fab)_2$ on G 200 Sephadex). Then separation of this population was achieved by density gradient. Sixty-seven per cent of T lymphocytes were recovered at a buoyant density of 1·072 and 1·095 (respectively 54% and 13%). These two sub-populations presented the same markers: EAC-Rosettes <5% and E-Rosettes > 80%. However, the response to mitogens was different. The 1·072 buoyant density cells responded to the three mitogens in the limits of the total purified T population. Contrary, the response of 1·095 buoyant density population was equal to 1·183 and that of the 1·095 buoyant density population was equal to 1·256 which is more rapid. Quite comparable results were achieved using a total lymphocyte population separated on BSA gradient purification. These results confirm the heterogeneity of the T-lymphocytes population probably related to different functions.*

INTRODUCTION

A NUMBER of studies in animals has demonstrated a functional heterogeneity existing within the T-lymphocyte population [1, 2]. Differences in response to the two mitogens, Phytohemagglutinin (PHA) and Concanavalin A (ConA), have also been reported for these sub-populations of T lymphocytes in animals [1]. It has been suggested that these differences in response reflected differences in maturation in T cells [1, 2]. It has been shown that there exists a distinct population of B and T lymphocytes in man [3, 4]. The studies of human sub-populations of type T and their differences in response to mitogens have drawn little attention until now [5]. In this paper we report results demonstrating the heterogeneity of human T lymphocytes with evidence showing differences in response to mitogens and electrophoretic mobility.

MATERIAL AND METHODS

1. *Extraction of lymphocytes and elimination of monocytes*

Human lymphocytes are obtained from whole blood centrifuged on a cushion of Ficoll–Hypaque according to the method of Boyüm [6]. Monocytes are eliminated by addition of iron carbonyl to the whole blood for 30 min at +37°C with continuous agitation; the monocytes having phagocytized the particles then traverse the cushion of Ficoll–Hypaque.

2. *Separation of T-lymphocytes*

We employ a modified technique of Schlossman and Hudson [7]. Briefly, a column of G-200 Sephadex, activated with cyanogen bromide and conjugated with anti-$(Fab)_2$ human serum, was prepared in a 20 ml syringe previously fitted with a nylon filter at the base and a 3-way stopcock. The column was freshly prepared and equilibrated with RPMI 1640 medium containing 1% Hepes, 5% fetal calf serum (FCS) and 2·5% EDTA (100 mM). This is designated complete medium and kept at +37°C until time of use. Lymphocyte concentrations were adjusted to $2 \cdot 10^7$/ml (total column population of 2×10^8) and are loaded on the column and eluted at the rate of 1 ml/mn. Elution was effected with 45 ml of complete RPMI 1640 medium.

3. *Separation of sub-populations of T-lymphocytes by discontinuous albumin (BSA) gradient*

We use BSA fraction V (Calbiochem) which was previously dialyzed, filtered and lyophilized. The dilutions at different densities (1·058, 1·064, 1·072 and 1·095) were adjusted by refractometer in Shortman's medium [8]. The dilutions of BSA were then sterilized, distributed in 1 ml aliquots and frozen. 10^8 lymphocytes were placed on the gradient and centrifuged for 30 min at $20{,}000 \times g$ at +4°C in an ultracentrifuge (MSE-25) using a swinging bucket rotor. Each lymphocyte band was then removed and washed in complete buffered medium. Viability was evaluated by the trypan-blue dye-exclusion procedure and the viable lymphocytes were then distributed for use in the various immunologic procedures.

4. *Immunological procedures*

E-Rosettes [9]. Sheep red blood cells (SRBC) were washed three times and adjusted to 350×10^6/ml. One then mixed 30 μl of lymphocyte suspension containing 5×10^5 cells, 30 μl of FCS previously adsorbed against SRBC and 60 μl of the sheep cell solution. The cells were then allowed to sediment for 5 min at $400 \times g$ and incubated at laboratory temperature overnight. The cells were then carefully resuspended in solution and the percentage of E-Rosettes was evaluated in duplicate.

EAC-Rosettes. 100 μl of lymphocyte suspension (2×10^6/ml) were added to 100 μl of the complex formed by the SRBC (10^9/ml) and rabbit anti-sheep erythrocyte serum (1/2000) in the presence of mouse complement (C57 B1/6 × DBA2)F1 (1/20). The mixture was then incubated for 5 min at +37°C, centrifuged for 5 min at $120 \times g$ and re-incubated for 30 min at +37°C. 200 cells were then counted in duplicate.

Surface immunoglobulin staining and monocyte peroxydase staining. A human anti-immunoglobulin serum (Institut Pasteur) was previously conjugated to fluorosceine isothiocyanate in the laboratory. 100 μl of lymphocyte suspension (2×10^6 cells) were mixed with 100 μl of serum previously conjugated with marker and this mixture was then incubated for 1 hr at +4°C. The cells were then washed 3 times in cold PBS containing 10% FCS. The cell suspension was then spread on a slide and those monocytes labeled with peroxydase were noted according to the method of Preud' Homme [10]. The lymphocytes were examined under the 25 × objective of an Orthoplan (Leitz) microscope equipped with a Ploem. 200 cells were counted in duplicate.

Blast stimulation of lymphocytes by mitogens. The blast stimulation of human lymphocytes has already been described [11]. Briefly, 20 ml of venous blood was collected in 20 ml of Hanks containing 0·2 ml of heparin. Seven ml of Ficoll-Metrizoate were placed at the bottom of each tube. After centrifugation at $400 \times g$ for 30 min, the lymphocyte band is washed three times in Hanks medium. Viability was controlled by the trypan blue dye exclusion procedure and the cell concentration was adjusted to 1×10^6 viable lymphocytes/ml. 10^5 lymphocytes in buffered medium 199 (20% AB serum) were placed in the wells of a microplate 3040 (Micro Test II; Falcon) followed by the appropriate mitogen (Phytohemagglutin, DIFCO: PHA-P; Concanavalin A, DIFCO: ConA; Pokeweed mitogen, GIBCO: PWM) in the respective concentrations of 75 μg/ml, 100 μg/ml and 75 μg/ml. The cultures were then incubated at +37°C in a humid environment for 72 hr. Sixteen hours prior to termination of incubation, 50 μl of tritiated methyl-thymidine were added to each well at a concentration of 4 μCi/ml (specific activity: 12–15 Ci/mM-CEA, Saclay, France). The cultures were filtered through glass fiber (FUG-France) with the aid of a semi-automatic harvesting collection apparatus (Mash 1–Otto–Hiller–Madison—U.S.A.). A count was made in a Beta counter SL-30 (Intertechnique).

Lymphocytes electrophoresis. Electrophoresis was accomplished by an analytical electrophoresis apparatus (cylindrical cell microelectrophoresis apparatus Mark 1, Rank Brothers). The lymphocytes were resuspended at 3×10^6/ml in PBS buffer, pH 7·2. The technique used was analogous to that of Wioland [12] and Sabolovic [13] with the following being held constant: electrodes Ag/AgCl; a constant voltage of 50 V; a constant temperature of +25°C; and glass capillary tubes of 2 mm dia. with an inner diameter of 100 μm. The slower moving lymphocytes were those which migrate at less than 1 μm $sec^{-1}V^{-1}cm$. The fast moving lymphocytes are those migrating above this value [13].

RESULTS

The experimental results show that populations 1·072 and 1·095 possess the same markers. The response to mitogens PHA, PWM, and ConA, on the contrary, is different. In effect, the population 1·072 responds as well to the three mitogens as the total non-separated population. If the population 1·095 responds in a sub-normal manner to the PHA, then there

is a marked reduction in response to PWM and ConA.

A study of the results of these two sub-populations obtained from T purified lymphocytes shows that the response of these lymphocytes to the three mitogens is very similar to those obtained from the total population. In effect, the 1·072 population responds to the three mitogens employed in a manner approximating the two sub-populations from total lymphocyte pools. On the other hand, a diminished response is seen for the 1·095 population for PHA, and moreover, there is a falling off in the response to PWM and particularly to ConA.

A study of the electrophoretic migration of these two sub-populations shows a different distribution of lymphocytes. The population 1·092 has a mobility equal to 1·164 while population 1·095 shows a mobility of 1·268, that is to say, a more rapid mobility.

Table 1. *For the three mitogens employed, the results are given as the relation of dpm of the sub-population studied* (1·072 *or* 1·095) *to the total non-separated lymphocyte population* (*T and B lymphocytes*). *A significant decrease is noted in the response of the sub-population* 1·095 *to PWM and ConA*

Subpopulations	PHA	PWM	ConA
1·072			
Exp. 1	1·29	ND	2·00
Exp. 2	3·55	1·30	2·63
Exp. 3	0·97	1·85	1·59
Exp. 4	1·08	1·31	1·33
Exp. 5	1·37	1·71	1·39
1·095			
Exp. 1	0·69	ND	0·05
Exp. 2	1·00	0·07	0·08
Exp. 3	0·26	0·33	0·15
Exp. 4	1·06	0·87	0·32
Exp. 5	0·91	0·37	0·10

Table 2. *A study of the markers employed shows no distinct difference between the* 2 *sub-populations. The presence of numerous monocytes is noted in the sub-population* 1.095 *which responds poorly to PWM and ConA*

Markers	Total lymphocytes	Subpopulations 1·072	1·095
Mean	63	78	67
E-rosettes			
% range	54–67	73–88	65–71
Mean	17	7	8
EAC-rosettes			
% range	14–21	7–9	6–11
Mean	14	2	21
peroxydase			
% range	11–16	0–4	16–26
Mean	—	69	10
Cell distribution			
% range	—	61–79	7–17

Table 3. *For the three mitogens employed, the results are given as the relation of dis/min. of the sub-population studied* (1·072 *or* 1·095) *to the dpm of the total purified T lymphocyte population. A weak response is noted for the* 1·095 *population to PM with an even weaker response to ConA*

Subpopulations	PHA	PWM	ConA
1·072			
Exp. 1	0·98	1·57	1·09
Exp. 2	0·72	1·33	1·12
Exp. 3	1·25	2·18	1·23
Exp. 4	0·92	0·69	0·44
Exp. 5	1·16	ND	1·05
1·095			
Exp. 1	0·87	0·63	0·40
Exp. 2	0·48	0·14	0·02
Exp. 3	0·62	0·28	0·08
Exp. 4	0·27	ND	0·04
Exp. 5	0·53	ND	0·04

Table 4. *The study of markers of the two sub-populations obtained upon separation from purified T lymphocytes confirms the results obtained from the two sub-populations obtained from the non-purified lymphocyte population. It is noted that monocytes are present in sub-population* 1·095 *which does not respond to ConA. These two sub-populations possess a different electrophoretic mobility, the sub-population* 1·095 *migrating more rapidly*

Markers	Purified T-lymphocytes	Subpopulations 1·072	1·095
Mean	84	88	82
E-rosettes			
% range	72–89	80–92	79–85
Mean	4	3	5
EAC-rosettes			
% range	3–5	2–4	3–4
Mean	2	0	3
Peroxydase			
% range	1–3	0	2–3
Electrophoretic mobility $\mu m\,sec^{-1}\,V^{-1}\,cm$	1·195	1·183	1·256
Mean	—	54	13
Cell distribution			
% range	—	50–80	5–27

The results obtained with the markers used do not give evidence of any distinct difference between populations 1·072 and 1·095 which would isolate them from the total mixed lymphocyte population or from a purified population of T lymphocytes.

DISCUSSION

The results presented suggest the existence of, at least, two sub-populations of T lymphocytes, one responding to all three mitogens, that is, PHA, PWM and ConA, and the other responding less to PHA, and very little to PWM and ConA. These two sub-populations exhibit identical markers, of those employed here, but possess different electrophoretic mobility, confirming therefore, that they behave as two sub-populations presenting distinctly different characteristics. In addition, this decreased response of the 1·095 lymphocyte population could not be associated with an absence of monocytes [14], because the monocytes present in the total population (B and T lymphocytes—when monocyte elimination procedure is omitted) are distributed for the most part in the sub-population 1·095, and these 1·095 lymphocytes do not respond to PWM or ConA. In spite of this, we do not observe a difference in response to ConA which varies from the response observed with sub-population 1·095 obtained from purified T lymphocyte population.

Of further interest, these two sub-populations possess a different morphology (R. Senelar, B. Serrou, C. Thierry, unpublished results) which should be noted. The lymphocytes responding well to the three mitogens are cells of greater diameter with remarkable cytoplasm possessing a number of ribosomes and mitochondria, while the cells not responding to ConA and PWM are the cells of smaller diameter with little cytoplasm as well as a number of lysosomes and Golgi apparatus worth noting.

It seems to us that these results should draw concern to the following: whether certain markers, such as those for the Fc receptor, may not be found to be associated with one or the other of two sub-populations, as might be suggested by the recent work of Stout *et al.* [15]. This would lead to the possibility that sub-population 1·095, which does not respond to ConA, would not be a sub-population of T Fc+lymphocytes. Be that as it may, we are presently following up this work by a study of the response of these two sub-populations in mixed lymphocyte culture and in the presence of antigens. The documentation of sub-groups of human T lymphocytes, possessing different functions, seems very interesting to us. The study of variations in distribution of these cells may, in effect, lend itself towards a better evaluation of the immune status of the cancer condition.

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Routine Technique of Lymphocyte Cryopreservation Evaluated by *In Vitro* Tests of Immune Response

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Abstract—*A reliable technique for cryopreservation of lymphocytes which conserves mitogenic response and surface marker (E-Rosettes and immunofluorescence) capability is presented. Briefly, lymphocytes are separated on Ficoll–Hypaque according to the method of Boyüm, washed twice. Concentration is adjusted and the cells are then placed in an ice-bath at +4°C in 7·5% Dimethyl sulfoxid (DMSO) and medium 199 supplemented with 20% AB serum. The cellular suspension is partitioned equally (1 ml containing 10^7 lymphocytes/vial) into freezing vials and placed in the chamber of a Planer R 201 Programmed Freezer with a cooling rate of 1°C/minute from +4°C to −60°C with rapid intense cooling between −6°C and −8°C to prevent surfusion and then a rapid gradient from −60°C to −160°C. Next, vials are transferred into a container of stored liquid nitrogen. At the moment of their employment, the cryopreserved lymphocytes are placed in a water-bath at +40°C, then diluted 10 fold within 2 min in culture medium 199 with 20% AB serum. Lymphocytes are counted, cell viability is evaluated by the Blue trypan dye exclusion test after which cells are distributed for diverse immunological tests. The data which is presented shows no significant difference between tests involving fresh and cryopreserved lymphocytes.*

THE USE of cryopreserved lymphocytes to follow the immunocompetence of cancer patients becomes absolutely indispensable if one wishes to take into account time variations in the treatment of the lymphocyte response of patients with diverse tumors. A systemic study of different lymphocyte cryopreservation techniques and their effect on measurable immunological parameters has been proposed during the last three years [1–8]. However, perfection of a dependable, reproducable cryopreservation technique, avoiding selection of a lymphocyte sub-population remains a problem still to be resolved.

This article delineates a cryopreservation technique respecting the surface markers and the capabilities of the lymphocyte response to mitogens as well as response to allogenic cells in mixed culture. In addition an excellent cryopreserved lymphocyte viability and satisfactory recuperation percentage would permit this method to pass from an experimental technique to a routine clinical procedure.

MATERIAL AND METHODS

Lymphocyte extraction

Peripheral blood from normal patients is collected in heparin (Calcium salt; Choay Laboratory), diluted 3 fold in Hanks solution (Pasteur Institute) containing 4% Hepes, 5% fetal calf serum (FCS; Gibco) and 80 μg/ml of Gentalline. FCS and Calciparine are present in the washing medium to avoid platelet aggregation.

The suspension is placed on a Ficoll–Metrizoat cushion (density 1·077) according to the method of Boyüm [9]. The lymphocyte band is removed, washed twice in Hanks solution after which the cells are counted and the concentration is adjusted to $2 \cdot 10^7$/ml in 199 Medium (Gibco) containing 4% Hepes, 5 units/ml of Calciparine and 20% fresh AB human serum without complement.

Freezing and thawing procedures

The cell suspension is placed in an ice bath

at +4°C. The cryopreservation medium (Dimethyl-Sulfoxide-DMSO) is then added drop by drop to an equal volume of the lymphocyte suspension. The frozen suspension has a final concentration of 7·5% DMSO, 10% in AB human serum and 10^7 cells/ml. 1 ml aliquots are distributed in freezing tubes (Polylabo Block Ref. 13001) which are placed in a programmed freezing apparatus which has been pre-cooled to +4°C (Planer R201).

Before the program is begun, a 5 min stabilisation period is necessary in order that the temperature in the programmed freezer and that in the freezing tubes are exactly the same. The freezing program is then initiated with a temperature descent gradient of 1°C/min from +4°C to −60°C, including a supplementary chilling between −6°C and −8°C in order to avoid temperature irregularities due to surfusion and then a rapid decline in temperature between −60°C and −150°C. We lower the temperature to −150°C in order to have a temperature safety margin when the aliquots are transferred from the programmed freezer to liquid nitrogen storage containers (RCB 60 Air Liquid).

The samples to be thawed are removed from storage and immediately plunged in an incubator at +40°C with constant agitation until the last crystal of ice has disappeared. The cells are then diluted 10 fold within 2 min in a 199 buffered medium containing 20% AB human serum and then washed twice. The lymphocytes are then counted, their concentration is adjusted and their viability is evaluated by the trypan blue dye exclusion procedure.

E-rosettes

Sheep red blood cells (SRBC) are collected in sodium citrate solution, washed 3 times in Hanks solution and adjusted to 350×10^6 cells/ml. 30 μl containing 5×10^5 lymphocytes are placed in contact with 30 μl of FCS previously adsorbed on SRBC and 60 μl of the SRBC suspension. The mixture is incubated overnight at room temperature after which the cells are gently resuspended. A cell count is performed in duplicate on 200 lymphocytes.

Surface immunoglobulin staining and monocyte staining

Human anti-immunoglobulin serum (Pasteur Institute) is conjugated with Fluorescein in our Laboratory. 100 μl containing 2×10^6 lymphocytes are placed in contact with 100 μl of Fluoresceine conjugated serum and allowed to remain at +4°C for 1 hr. The cells are washed three times in cold PBS containing 10% FCS. The cell suspension is spread on a slide followed by a peroxidase labelling procedure for monocytes as described by Preud' homme [10]. Next, the lymphocytes are examined in a 25× objective of an Orthoplan (Leitz) microscope equipped with a Ploem. 200 cells are counted in duplicate.

Lymphocyte stimulation by mitogens

We use a method previously described [11]. Briefly, 100 μl of the lymphocyte suspension adjusted to 1×10^6/ml is placed in the wells of a microplate (Microtest II—Falcon) and 50 μl of the appropriate mitogen is distributed to its respective lymphocyte sample. The microplates are uncovered by a Blenderm (3M Company) and placed at +37°C in a humid atmosphere during 72 hr. 16 hr before termination of the culture, 50 μl of tritiated Methyl Thymidine (CEA, Saclay, France; specific activity of 15 Ci/mM) containing 0·2 μCi are added to the culture. The cultures are then filtered through fiber glass (Fug, France) with the aid of a multiple automated sample harvester (Mash 1, Otto-Hiller), dried, and then counted in a liquid scintillation counter (SL 30 Intertechnique). The results are evaluated statistically on a Multi 4 Computer (Intertechnique) and are expressed in dis/min ± 2 standard errors (SE).

Mixed lymphocyte culture

We used a microplate (Microtest II, Falcon) technique. The stimulating cells are blocked with Mitomycin C (Sigma; 8 μg/2×10^6 cells) for 30 min at +37°C with constant agitation, after which the cells are washed three times in buffered medium 199. A ratio of 1/1 (i.e. $5 \times 10^5/5 \times 10^5$ stimulating cells/cells stimulated) is used after prior experimental determination of the optimum cell ratio. Culture time was five days. 50 μl of tritiated methylthymidine (4 μCi/ml; specific activity of 15 Ci/mM; CEA, Saclay, France) is added to the cultures 16 hr prior to termination of incubation. Cell collection is effected with the aid of a Mash 1 and the lymphocytes are recovered by filtration through fiber glass. Radioactivity is monitored with a Beta scintillation counter (Intertechnique). Counts are expressed in counts/min ± 2 standard errors.

RESULTS

At the outset, we experimented with 4 freezing protocols: progressive freezing in liquid nitrogen vapor; a programmed decrease in temperature of 0·6°C/mn to −25°C; a programmed decrease of 1°C/mn to −25°C and

lower; a programmed decrease of 1°C/mn to −15°C and 3°C/mn to −25°C. In addition we have also varied the DMSO concentration. We also compared fresh AB serum kept at a maximum of +4°C for 10 days to frozen AB serum.

The cryopreserved lymphocytes under these conditions have been tested in mixed lymphocyte culture. As shown in Fig. 1, protocol 1 was rejected, the results being uninterpretable. On the other hand, protocol 3 (i.e. decrease in temperature of 1°C/mn to −25°C and lower) yielded the optimum results with a DMSO concentration of 7·5%. Use of fresh or frozen AB serum yields no significant difference in the results.

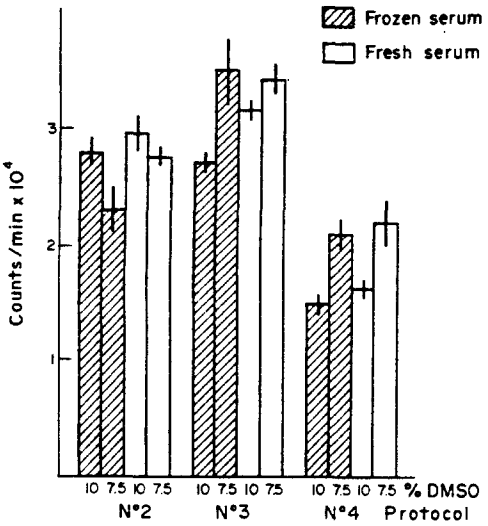

Fig. 1. Comparison of 3 different protocoles of lymphocyte cryopreservation evaluated by the mixed lymphocyte culture. Results are expressed in Counts/min × 10⁴ lymphocytes. Note that the best results are obtained using 7·5% DMSO with the protocol 3 (1°C/min. up to −25°C and minus). The results are similar for fresh or stored AB human serum.

Within the context of these results, we have defined a technique which we are presently employing. Figure 2 shows the freezing curve used which is scrutinized, in the upper and lower regions, in the following manner: stabilization for 5 min at +4°C followed by a programmed decrease in temperature from +4°C to −60°C with intense cooling between −6°C and −8°C, which permits us to obtain a curve approximating linearity. Between −60°C and −150°C the descent is rapid.

As shown in Table 1, the surface markers of fresh and cryopreserved lymphocytes are studied comparatively. The viability of cryopreserved lymphocytes varies between 75% and 92% while the percent recovered is between 42% and 89% with an average of 65%. On the other hand, during the course of 14 experiments

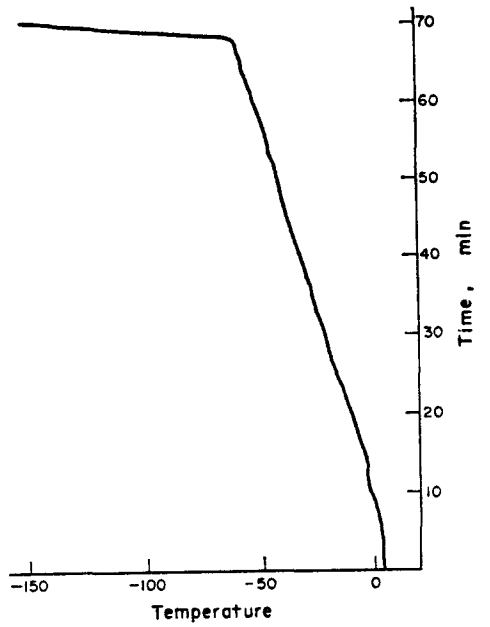

Fig. 2. Temperature curve of lymphocyte cryopreservation used in this work. Note the absence of surfusion phenomenon on this curve due to the intense cooling used between −6°C and −8°C.

performed, the average difference between the fresh and cryopreserved lymphocytes was 5% for the E-Rosettes, 2% for immunofluorescence and 2% for the peroxidase monocyte marker.

Figure 3 shows the distribution of results which we have obtained by comparison of fresh lymphocytes and those cryopreserved, both having been stimulated to a blastic response by Phytohemagglutinin (PHA-A), pokeweed mitogen (PWM-B) or Concanavalin A (ConA-C). Taken together the results are very similar and the average values for fresh and cryopreserved lymphocytes are very close. There is no significant difference. It should be noted that there is a wide dispersion in the results observed for the response to PWM.

DISCUSSION

A number of studies using different protocols to decrease the temperature (1–5°C/mn) as well as employing different concentrations of DMSO (5–10%) have obtained satisfactory results [4, 7, 12, 13]. Studies of the ability of cryopreserved lymphocytes to respond to PHA, PWM and ConA as well as mixed culture procedures have been published with a certain heterogeneity in the results [4, 6, 7, 12, 13]. In addition, certain investigators have proposed the possibility that cryopreservation results in selection of lymphocyte sub-populations [6, 14].

The technique of cryopreservation which we

Table 1. *Comparison of the surface markers between fresh and cryopreserved lymphocytes. No significant difference was observed*

a: *E. Rosettes Test.*
b: *Surface immunoglobulin staining*
c: *Monocyte staining by peroxidase*
ND: *Not Done*

	Fresh lymphocytes			Cryopreserved lymphocytes				
Exp.	E.R.(a) (%)	I.Fl. (b) (%)	Mono. Peroxid. (c) (%)	Viability (%)	Recovery (%)	E.R. (%)	I.Fl. (%)	Mono. Peroxid. (%)
1	75	14	5	82	89	77	15	3
2	72	9	11	84	63	70	9	11
3	74	9	4	90	80	74	7	10
4	53	18	6	82	77	48	12	8
5	63	19	22	86	67	57	12	24
6	46	7	8	82	62	55	7	8
7	64	13	7	76	87	63	13	7
8	66	14	8	85	57	61	16	6
9	48	17	13	88	43	51	14	16
10	60	10	20	92	56	52	7	9
11	66	10	3	75	55	70	10	4
12	42	11	ND	86	42	57	10	ND
13	59	15	ND	86	42	57	18	ND
14	60	12	ND	86	69	64	12	ND

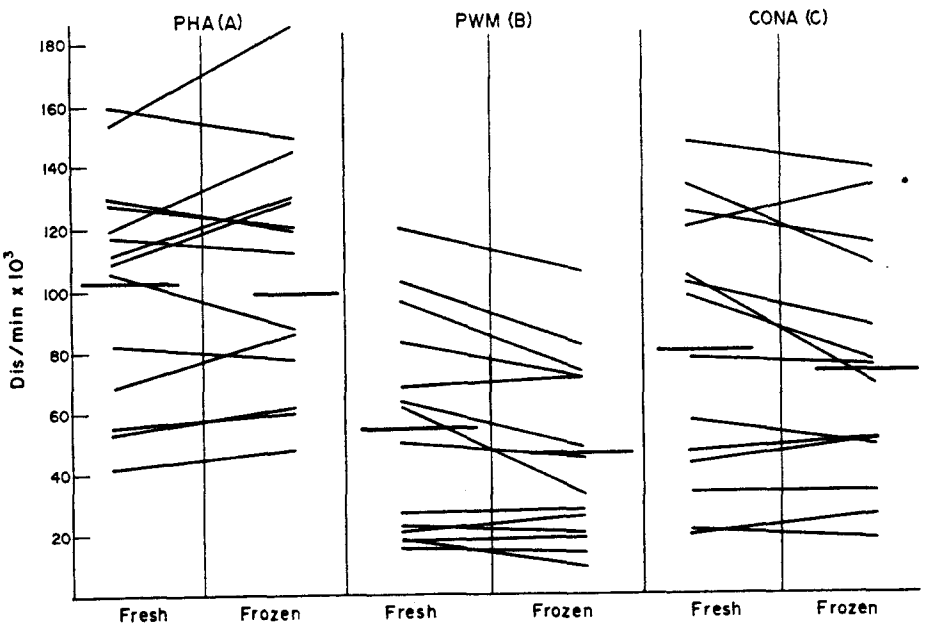

Fig. 3. *Comparison of mitogen response between fresh and frozen lymphocytes. The mitogen dose used for the phytohemagglutinin (PHA-P, Difco;A) was* 75 *μg/ml, for the pokeweed mitogen (PWM, Gibco; B)* 70 *μg/ml and for Concanavalin A (ConA, Difco; C)* 100 *μg/ml. Results are given in dis/min. Note the homogeneity of the results obtained.*

have presented seems feasible and reproducable. In addition, the results which we have obtained by evaluation of different markers has allowed the *a priori* rejection of subpopulation selectivity. In the time, considering the dispersion of certain results of blastic stimulation by mitogens and the relative percent recovery, we have not yet reached a definitive position. In spite of this, we think that this technique is presently capable of being employed routinely in the immunological laboratory wishing to preserve lymphocytes under the best conditions possible for study of the immunological balance in the tumour patient.

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Carcino-Embryonic Antigen Assay in Breast and Bronchus Cancers

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INTRODUCTION

MANY teams have focussed their attention on carcino-embryonic antigen (CEA) in colonic carcinoma, since its discovery in 1965 by Gold and Freedman [1], Koldovki [2] published a general review concerning this antigen. Since its radioimmunoassay was introduced by Thomson *et al.* [3] then by Martin *et al.* [4], CEA has been assayed in many other localisations, then in colonic carcinoma. This work concerns bronchus and breast cancers.

MATERIAL AND METHODS

(1) The CEA radioimmunoassay has been established thanks to reagents commercialized by the "Commissariat de l'Energie Atomique", Henry *et al.* [5].

(2) *Populations*

(a) 45 assays were performed on 45 bronchus cancer patients, of which 29 were done pre-therapeutically.

(b) 87 assays were performed on 87 breast cancer patients of which 54 were pre-therapeutic.

(c) 48 women were followed up periodically (248 assays).

RESULTS

(1) *Pre-therapeutic levels in bronchus cancers*

Generally pre-therapeutic levels are higher than post-therapeutic ones; nevertheless, low values can be observed ranging from 0 to 800 ng/ml.

Figure 1 shows that there is no relation between primary tumour size and CEA blood level. But this relation does exist as has been shown by several authors [6–8] between blood levels and lymphnode-involvement or metastatic spread.

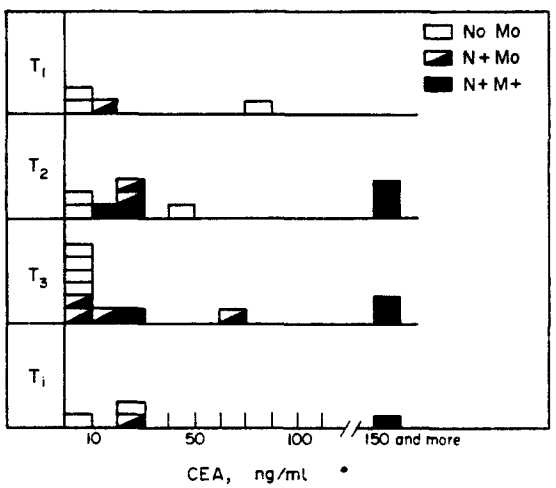

Fig. 1. Pre-therapeutic CEA levels in patients bearing bronchial carnoma with special reference to tumour size, lymph node involvement and metastatic spread.

(2) *CEA levels in breast cancer*

Figure 2 shows, once more, that primary tumour size has no influence on CEA blood level.

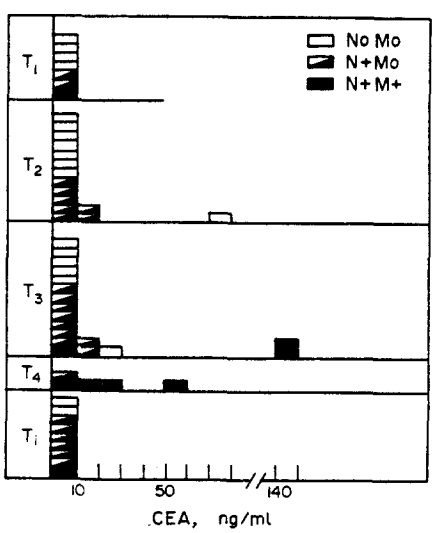

Fig. 2. Pre-therapeutic CEA levels in patients bearing breast cancers with special reference to tumour size, lymph node involvement and metastatic spread.

However, lymphnodes as well as metastases at distance (Table 1) are responsible for CEA blood level increase.

Table 1. *Percentage of plasma CEA levels above* 5 *ng/ml in bronchus and breast cancers*

	Bronchus carcinoma	Breast cancer
NO MO	21	11
N +MO	61	33
M +M+	95	65

This table shows the relative increase of blood levels above 5 ng/ml according to clinical status.

(3) *Follow up of* 48 *breast cancer patients*

Different types of evolution in CEA blood level has been observed.

DISCUSSION

Studies made on patients using one form of assay show an obvious relationship between CEA blood level and clinical extension of disease. They also show that breast cancers produce less CEA than bronchial carcinomas.

But one assay is insufficient [6, 8] and follow up greatly improves the information gained about the marker's behaviour characteristics. Effectively, only one patient (Fig. 3) has an interpretable kinetic.

CONCLUSION

In these series, the radioimmunoassay of carcino-embryonic antigen especially during long term follow-up appeared to be a good indicator of prognosis and of metastatic dissemination.

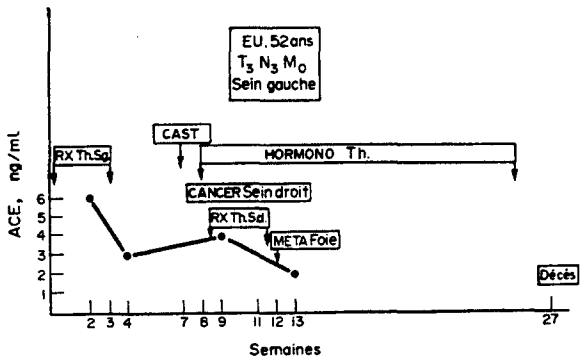

Fig. 3. *Mme EU . . .* (52) *has never shown CEA increase despite bone metastatic complications and control-lateral cancer appearance followed by rapid death.*

Table 2.

Type of kinetic and number		Clinical evolution: Good	Stable*	Death
Increasing levels	8			8
High levels	2			2
Decreasing levels	9	8		1
Low levels (less than 5·0 ng/ml)	18	18		
Fluctuating levels (between 0 and 10 ng/ml)	8	8		
Fluctuating levels (between 10 and 30 ng/ml)	3		3	

*Presence of metastases but without evolution.

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An Effective Human Leukaemic Cell Line: Reh*

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Abstract—*We report here an unusual cell line (Reh) initiated from the peripheral blood of a patient with acute lymphoid leukaemia. This line differs from the data we reported previously for other cell lines by its kinetics of establishment, by chromosome markers found on fresh and established cells, by the absence of EBNA and the presence of EBV receptors and other B markers.*

Reh cells are presently utilized for active immunotherapy of patients with acute lymphoid leukaemia.

INTRODUCTION

NUMEROUS lymphoblastoid cell lines have been initiated from the peripheral blood of patients with malignant disease or from healthy donors [1]. Establishment usually occurs after a lag phase of variable duration where neither mitosis nor DNA synthesis can be observed [2–3]. In nearly all cases these lines contain Epstein-Barr virus (EBV) or some antigens determined by the genome of the virus [4], which can be detected in the cellular DNA [5]. All these cell lines demonstrate B cell markers [6]. Very few exceptions have been found, namely T cell lines lacking EBV [7]. More recently Klein and his co-workers have reported B cell lines without the EBV genome and the Epstein–Barr determined nuclear antigen (EBNA), established from biopsies of African or American lymphosarcomas [8].

From this data, it has been claimed that all categories of cell lines originate from a small number of primitive stem cells infected by EBV [1].

However, differences between normal and leukaemic cell lines have been published [9–11].

We report here a cell line initiated from a leukaemic donor which differs from the commonly described lymphoblastoid cell lines by its kinetics of establishment, by chromosome markers already present in leukaemic fresh cells, the absence of EBNA, and the presence of EBV receptors and other B. markers.

MATERIAL AND METHODS

The cells were handled following a procedure previously described [12]. Briefly, they were cultivated in RPMI 1640 medium supplemented with fetal calf serum and antibiotics. The cumulative growth curve has been obtained by plotting the total cell number found in each flask on semi-logarithmic paper. Once established this curve was made by multiplying the ratio between the number of cells at the time of transfer and the respective inoculum by the preceding cell number on the curve [3].

Chromosome studies were performed using the method of Lejeune [13]. At the time of initiation of the culture, the blood cells were cultivated during 24 hr and 48 hr without PHA.

EBV rosettes: the technique described by Jondal and Klein [14] was employed. Briefly 10^5 P_3 HR-1 producing cells were labelled with 50 μl of fluorescein conjugated MA positive antiserum (Tu 125). After two washes with cold BSS the labelled cells were resuspended and incubated at 4°C for one hour. in the presence

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of $1 \cdot 10^6$ cells to be tested. The mixture was then gently resuspended. One hundred MA positive P_3 HR-1 cells were examined alternatively in UV and ordinary light and the percentage of producing cells forming rosettes was determined.

EAC rosettes. The formation of EAC rosettes was assayed by the method of Shevach *et al.* [15]. Rabbit antisheep red blood cells were purchased from Gibco. Fresh AkR serum, diluted at 1/20 was used as source of complement.

EBNA determination. Detection of EBNA was performed by anti-complement immunofluorescence according to Reedman and Klein [4].

RESULTS

The cell number, which was initially of $1 \cdot 2 \times 10^8$, did not decrease below 1×10^8 (Fig. 1), with continuous multiplication compensating for cell death (to be published).

Cytogenetic examination at day 0 revealed the presence of aneuploid cells with a 45, XX, −2B, +1C karyotype. Four months later, the whole population of the established cells exhibited this same abnormal karyotype.

It has never been possible to demonstrate the presence of EBNA, even after treatment by Iodo-deoxy-Uridine. Nevertheless, it was possible to show the presence of EBV receptors by the rosette's technique. Approximately 70% of EBV producing cells, P_3J HR-1, formed rosettes with Reh cells. This marker is considered to be a property of B cells [14]. Other B cell markers have been detected such as membrane immunoglobulins and C_3 receptors (to be published).

DISCUSSION

This line is clearly the offspring of the abnormal leukaemic cells from the patient. It demonstrates characteristic features such as proliferation from the beginning of the culture, which differentiates it from other lymphoblastoid cell lines. In this particular case, the establishment could result from the association of immediate proliferation with progressive adaptation of the initially abnormal cells. Such a mechanism would differ from the usual mode of establishment where transformation *in vitro* seems to be a prerequisite of cell multiplication [3, 12].

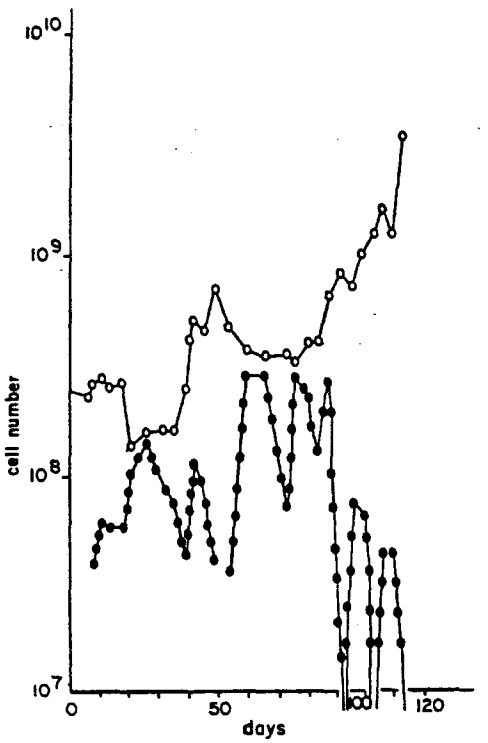

Fig. 1. Growth curve of the human leukaemic cell line: Reh

○——● *living cells*
○——● *dead cells.*

Other characteristics have never been described in leukaemic cell lines which lack EBNA. Until now the few leukaemic cell lines without EBNA demonstrate T type markers [4, 16]. Only exceptional lymphoma cell lines, lacking EBNA, and bearing some B cell markers, have been described before now [8].

We are currently studying some immunological and biochemical properties and the evolution of the cells of this line.

Data already obtained allowed us to utilize Reh cells for active immunotherapy of patients with acute lymphoid leukaemia [17].

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Anaplastic Carcinoma of the Thyroid Gland

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Abstract—*Thirty cases of anaplastic thyroid carcinoma are reviewed. Clinical and pathological characteristics are briefly reported. Prognosis is very bad: only 1 patient survived more than 2 years and treatment (surgery and radiotherapy) is rarely efficient; Adriamycin, used in 5 more recent cases, gave one good remission and has to be used in all cases in association with other treatments.*

INTRODUCTION

ANAPLASTIC carcinomas of the thyroid gland appearing in old people grow rapidly and are fatal. They are opposed to well differentiated carcinomas of younger people, which have a slower evolution and well defined treatment. The frequency of the anaplastic type is about 20–25% of all thyroid carcinomas.

MATERIAL AND METHODS

We present observations of anaplastic carcinomas collected at the Centre Regional de Lutte contre le Cancer de NANCY: 30 between 1957 and 1973 and 5 more recent but which concern patients having received adriamycin. During this period, 144 thyroid carcinomas were observed, anaplastic type representing 20%. All these cases have been reviewed by the same pathologist (Dr. Parache).

Pathological aspect

These carcinomas were classified in 2 groups:

(1) Small cell carcinoma (9 cases) with round cells of the same size as lymphocytes, acidophylic cytoplasm, well delimited. There are many mitoses and frequent nuclear abnormalities: the nucleus is round or oval, rich in chromatin, with one or more nucleols. These are either grouped, in dense accumulation, divided by fibrous septa or spread without order, diffusely. The distinction with malignant lymphomas is often difficult.

(2) Giant cell carcinoma (20 cases)

They have a pleomorphic appearance and we can see:

spindle-shaped cells,
giant cells with several nuclei,
polygonal or round great cells, with abundant cytoplasm and hyperchromatic nuclei.

(3) Other aspects

In one observation, the carcinoma was mixed with small and spindle-shaped cells.

In another case, we noted the association of a well differentiated carcinoma with an anaplastic one. One time the metastases were well-differentiated (vesicular in the liver) and the primary was a giant cell anaplastic type.

Clinical data

The extreme ages at time of diagnosis were 30 and 87 (average 66). Sex ratio showed 27 women for 3 men. The small cell type was observed only in women.

Pre-existent goitre was found in 16 cases for at least 4 years (maximum 19 years).

One patient, a 72-year-old woman, was irradiated 35 years before diagnosis for a simple goitre by external radiotherapy.

None of our patients received I131 before diagnosis. Seven patients received iodine or antithyroid drugs many years before diagnosis.

The beginning of the symptoms is usually sudden. Time between the first symptom and the diagnosis is 3 months in average. The first symptoms are:

appearance of a goitre or modification of an old one (2 cases)
cervical or mediastinal compression: dyspnea, dysphagia, dysphonia, pain
asthenia, loss of weight
cervical lymph node involvement (17 cases)
metastatic disease (7 cases, essentially pulmonary).

We never observed dysthyroidism: biological and isotopic examinations were normal or subnormal. Inflammatory signs were often present. Anti-thyroid anti-bodies were found in 3 cases on 6 studied. Thyroid scan showed no fixation on the tumoral region. Metastases never fixed I131.

Table 1. *Summary of observations*

No.	Age	Sex	Old goitre	Pathology	Treatment	Survival	
						From 1st symptom	From diagnosis
1	82	F		Small cells	RX	5 months	3 months
2	74	F	44 years	Giant cells	RX	3 months	2 months 1/2
3	74	F	10 years	Small cells	Lobectomy + Neck dissection	12 months	11 months
4	77	F	old	Giant cells	Thyroidectomy, RX	5 months	3 months
5	30	F		Small cells	Thyroidectomy, RX	20 months	18 months
6	60	F	6 years	Giant cells	Lobectomy + Neck dis. RX	5 months	4 months 1/2
7	52	F		Small cells	RX, Chemotherapy	2 months	1 month
8	71	F		Small cells + Giant cells	Lobectomy, RX, Chemotherapy	14–18 months	12 months
9	61	F	old	Small cells	RX	26 months	20 months
10	61·5	M		Giant cells	RX, Chemotherapy	6 months	5 months
11	64·5	F	old	Giant cells	Lobectomy + Neck dis., RX Chemotherapy	17 months	16 months
12	69	F		Giant cells	Lobectomy + Neck dis., RX	6 months 1/2	6 months
13	86	F		Small cells		3 months	6 days
14	72	F		Giant cells	Tracheotomy, RX, Chemotherapy	8 months	6 months
15	64	M		Giant cells	T.T., RX, Chemotherapy	8 months	6 months
16	63·5	F		Giant cells	Tracheotomy, RX	4 months	1 month 1/2
17	55·5	F	15 years	Giant cells	RX, Chemotherapy	6 months	4 months 1/2
18	18	F	32 years	Giant cells	Incomplete thyroidectomy	2 months	1 month
19	58·5	F	old	Giant cells	RX, Chemotherapy	5 months	3 months 1/2
20	58·5	F	19 years	Giant cells	RX, Chemotherapy	6 months	5 months
21	75	F	30–40 years	Giant cells	Tracheotomy, RX	4 months	2 months
22	79·5	F		Small cells	RX	5 months	2 months
23	44·5	M		Giant cells	Incomplete Treatment (?) RX, Chemotherapy	18 months	11 months
24	68	F	4 years	Giant cells	RX, local chemotherapy	1 month	18 days
25	67·5	F	old	Giant cells	RX	6 months	3 months 1/2
26	65·5	F	10 years	Giant cells	RX	4–5 months	2 months 1/2
27	69	F		Giant cells	RX, Chemotherapy	9 months	5 months
28	69	F		Giant cells	T.T., Tracheotomy, RX, Chemotherapy	3 months	1 month 1/2
29	87	F		Small cells	RX	2 years	alive
30	69	F	4 years	Small cells	RX	8 months	6 months

RESULTS

The prognosis was very bad: average survival of the serie is 8·4 months (11 months for small cells carcinomas and 7·2 for giant cells carcinomas). Only 33% were alive after 6 months and 25% one year after the diagnosis.

The cause of death was local evolution in 75% and metastatic disease in 25%.

Treatment seemed to have a low influence on the survival:

Surgery was done in 13 cases.

4 total thyroidectomies (maximum survival of 6 months),

5 lobectomies with or without neck dissection (maximum survival of 16 months),

4 cases: palliative surgery (tracheotomy and biopsy).

External irradiation was done in 28 patients. A good tumor response (regression of more than 50% of the initial volume) was observed in 50% of patients who received more than 4000 rads. One patient is in total remission at 2 years without metastases.

I131 was used in one case without effect.

Chemotherapy: multiple drugs treatments were used on one third of the patients: no objective result was noted. Adriamycin was used more recently. Five patients, out of the present study received this drug (Table 2). There was an objective remission in only one case: the patient is still alive 6 months after irradiation and adriamycin treatment, but with a residual tumor.

Table 2. Results of therapy with adriamycin

Age	Sex	Pathology	Adriamycin dosage (mg)	Survival	Objective result		
					>50%	<50%	Unsuccess
72	F	S.C.	215	6 months alive	+		
40	M	G.C.	340	6 months			+
64	F	S.C.	420	4 months		+	
66	M	G.C.	400	3 months alive			+
72	F	G.C.	400	11 months			+

DISCUSSION

The study of this group of patients shows the usual characteristics of anaplastic carcinoma of the thyroid gland. According to publications, we find patients 60 or over, with old goitre which grow suddenly and rapidly, compressing neighbouring organs.

Pathological studies show 2 groups: giant cell carcinomas which when they are of spindle-shaped type must be distinguished with sarcoma and small cells that must not be confused with malignant lymphomas. Histogenesis from well differentiated carcinoma is supposed.

Prognosis stays very bad and, if surgery is efficient in the rare little tumor, I131 is always inefficient and external radiotherapy can sometimes give a remission in the small cell type. With chemotherapy, especially adriamycin, good remissions have been reported, and this treatment has to be used in all cases with surgery or radiotherapy. When a spectacular remission is observed with adriamycin or radiotherapy, the diagnosis of lymphoma can be discussed.

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The Clinical Significance of Skeletal Scintigraphy in the Management of Carcinoma

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Abstract—*The majority of skeletal metastases originate via localization in the bone marrow. A study of the various $^{99}Tc^{m}$ polyphosfate complexes available for skeletal scintigraphy suggests that the use of short chain polyphosphates with a significant bone marrow uptake is advantageous in detecting skeletal metastases at an early stage.*

MATERIAL AND METHODS

THERE is little doubt that the introduction of skeletal localizing technetium compounds has made a significant contribution to the early diagnosis of pathological changes involving the skeleton. There are a number of complexes in current use and with all of them it has been possible to detect malignancy several months before they become visible to X-ray investigation [1].

Work in our laboratories has shown that the degree of localization in the skeleton is variable and depends upon the nature of the phosphate used. Phosphates with a strong chelating effect, such as diphosphonate, pyrophosphate and monofluorophosphate are rapidly cleared from the blood and localize primarily in bone. Others, such as the short chain polyphosphates also localize to a high degree in bone, but also have a significant uptake in bone marrow [2].

Hashimoto [3] suggested that 80% of bone metastases originated via the bonemarrow and therefore it would appear that the use of polyphosphate with a high degree of bonemarrow uptake should be advantageous in detecting malignancy in comparison with the more bone specific complexes. A comparison of polyphosphate localization in skeletal metastases with pyrophosphate, which has a low bonemarrow uptake, clearly showed a significant difference in the number of lesions detected, the pyrophosphate visualizing 23% less lesions [5]. This would appear to be of some significance since it has been shown that the diagnostic accuracy of polyphosphate scintigrams, in the absence of other clinical signs of malignancy, is in excess of 90% [6].

In this presentation a retrospective review has been made of 259 polyphosphate scintigrams. These have been classified according to the nature of the primary diagnosis (Table 1). For the scintigraphic study 8 mCi $^{99}Tc^{m}$ polyphosphate (Diagnostic Isotopes Inc.) was administered intravenously three hours prior to preparing scintigrams by means of a Nuclear Chicago HP gamma camera. This polyphosphate has a molecular weight of 4–6000 and a bone/bonemarrow uptake ratio of 4:1.

In Table 2 the number of patients with positive scintigrams has been compared with the X-ray examinations. Table 3 shows the results obtained with 118 of the patient samples, who presented with skeletal associated pain as the only clinical sign of metastases. In 5 cases the scintigram was negative and the X-ray positive. Information on these cases is given in Table 4.

DISCUSSION

These first retrospective studies suggest that Tc-polyphosphate complex is a reliable reagent for the early detection of skeletal metastases

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Table 1. Patient classification according to the primary diagnosis

Primary diagnosis	Number of cases
Head and neck tumours	2
Seminoma testis	3
Lymphoma	4
Breast carcinoma	104
Lung- and bronchuscarcinoma	49
Carcinoma of the digestive tract	19
Bladder carcinoma	12
Renal carcinoma	6
Prostate carcinoma	21
Carcinoma of the cervix uteri	8
Endometriumcarcinoma	1
Reticulosarcoma	6
Paget's disease	1
Rheumatoid arthritis	1
Melanoma	1
Skin cancers	1
Sarcoma	3
Morbus Kahler	1
Thyroid carcinoma	9
Unknown aetiology	3
Ovarium carcinoma	4
	259

Table 2. A comparison of scintigraphic results with X-ray results

Result	Positive	Dubious	Negative
Skeletal scintigram	120	75	58
Skeletal X-ray	41	21	197

Table 3. Verification of scintigram by follow up in patients with known primary tumours and skeletal associated pain as the only visible symptom of metastatic disease

Scintigram	Positive	Doubtful	Negative
Subsequent identified Skeletal lesions on X-ray	49 / 10	44 / 5	14 / 1
Number of scintigrams verified by follow up (X-ray cytology etc.)	92%	60%	86%
Not verified (possible faulty diagnosis)	8%	14%	14%

Table 4. Verifications negative scintigrams with positive X-ray examination

Scintigrams negative	X-ray positive	Verification
1	1	surgical examination negative for metastases.
1	dubious	post mortem negative.
2	2	not verified.
1	1	irradiated area (see text).

with certain advantages over more bone specific reagents such as pyrophosphate. The diagnostic value in comparison with X-ray is obvious (Table 2) and the degree of accuracy is high (Table 3).

In the few cases with negative scintigrams and positive X-rays the scintigram result was substantiated in 2 cases and not verified in 2 cases. In the fifth case metastatic disease was demonstrated, but the lesion had been irradiated prior to scintigraphy. [7] demonstrated that irradiated regions show reduced $^{99}Tc^{m}$ complex uptake, which explains the negative scintigram.

In clinical terms $^{99}Tc^{m}$ polyphosphate scintigraphy can be used to detect skeletal metastatic disease with a high degree of reliability. In general it was found that lesions could be detected on an average 6·2 months before the X-ray became positive (σ 4·4). The early detection of skeletal lesions facilitates therapy planning and the sensitivity of the technique appears to make the possibility of evaluating the response to therapy. The response of irradiated regions has been clearly demonstrated [8], [7] and we have also observed similar response to steroid therapy.

A typical example of the latter is shown in Fig. 1. In this patient a reduction in polyphosphate uptake following Lynoral treatment can be seen. That this is a positive response of the lesions to the treatment is shown by the fact that new lesions can be seen to be developing in the follow up scintigrams, so that the reduced uptake in the old lesions can only be explained by a therapeutic response.

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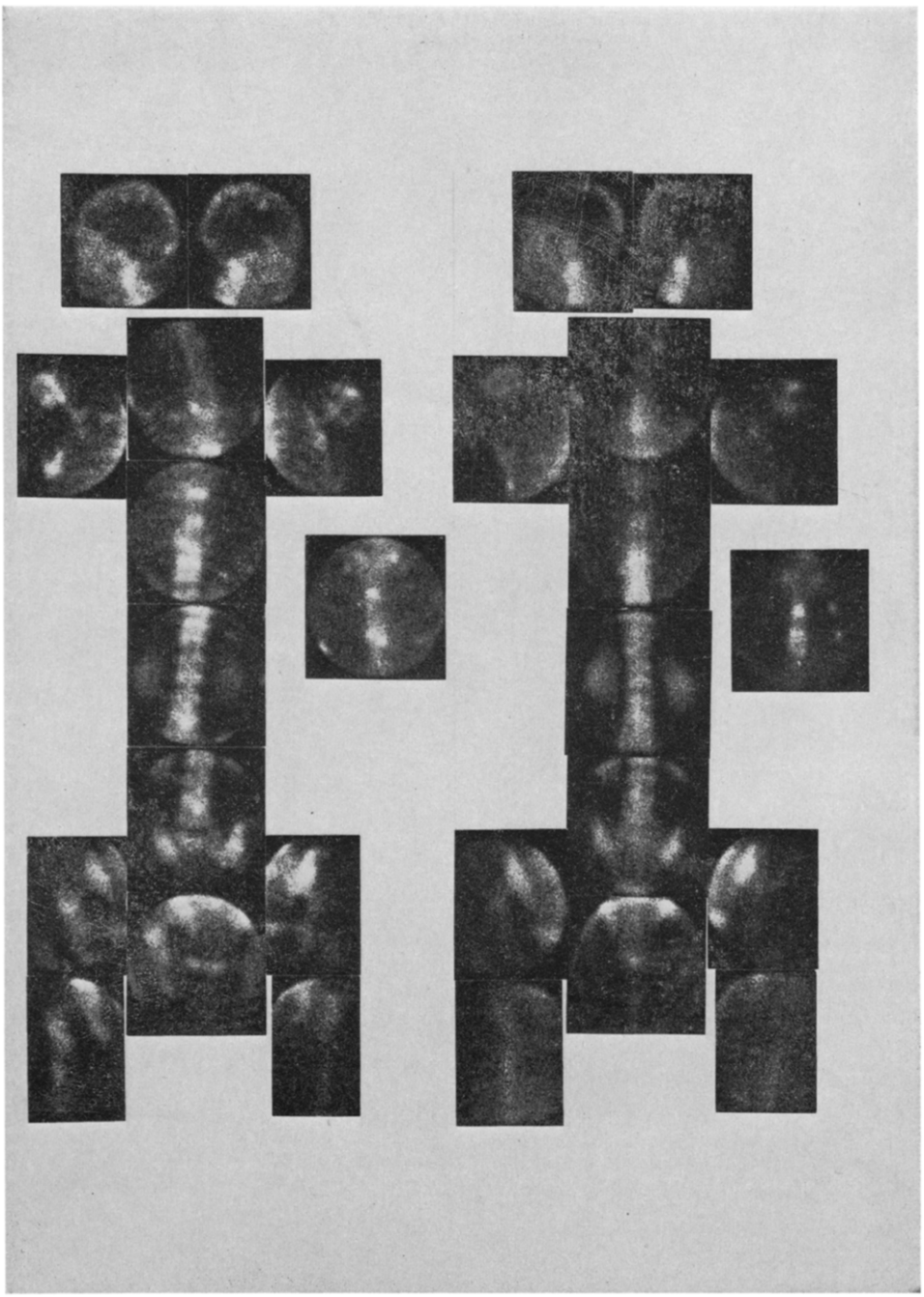

Fig. 1. *Polyphosphate skeletal scintigrams of a patient with multiple metastases from a breast carcinoma before (left) and after (right) ethinyl oestradiol therapy. In the right hand scintigram the effect of therapy can clearly be observed together with the development of new lesions.*

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Therapeutic Effect of Prednimustine (LEO 1031) in Various Types of Leukaemia

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Abstract—*Prednimustine, a chlorambucil ester of prednisolone, is known to be effective in the treatment of chronic lymphocytic leukaemia. In a pilot study the drug was administered to seven patients more than 60 years old (mean 71) with acute myeloid leukaemia (AML). Complete remissions were obtained in two patients. Prednimustine was also given in combination with vincristine to four adults younger than 60 years (mean 43) resulting in two complete remissions. One patient with acute lymphocytic leukaemia also obtained a remission with this treatment. The remission was not preceded by a drug-induced pancytopenic phase in any of these five patients.*

INTRODUCTION

PREDNIMUSTINE, a chlorambucil ester of prednisolone [1] is effective with high therapeutic indices in several animal tumours [2]. It is known that the killing effect on L1210 leukaemia cells compared to the effect on normal resting haematopoietic cells is superior to any other alkylating agent tested in this system [3]. The drug is of value in the treatment of human chronic lymphocytic leukaemia (CLL) [4, 5]. In the present report it is demonstrated that Prednimustine may be useful also in the treatment of acute leukaemia.

It has been stated that patients with acute myeloid leukaemia (AML) have to survive a period of drug-induced bone marrow hypoplasia and pancytopenia before remission can be obtained [6–8]. Current intensive treatment of acute leukaemia in adults will give an overall remission rate of about 50–60% [7]. In patients over the age of 60 a remission frequency of about 15–30% and a median survival time of only 0·5–2 months have been reported [9–13].

With the above considerations in mind we initiated a pilot study in AML patients over the age of 60. It was decided to postpone chemotherapy until a pronounced need for transfusions arose, or neutropenia with infections or thrombocytopenia with a tendency to bleeding, justified an attempt to induce a remission. Moreover it was considered justified to try a chemotherapy regimen known to be associated with a minimum of toxicity to normal haematopoietic tissue. Prednimustine was therefore given in doses known to have an antitumour effect without serious toxicity to normal haematopoietic tissue in lymphocytic lymphoma [4] and in mammary carcinoma [15].

Since it soon became evident that the drug can induce remissions in elderly patients with AML the pilot study was extended to include also younger adults with AML or acute lymphoblastic leukaemia (ALL). Because there is evidence that younger patients may generally tolerate combination chemotherapy better than the older ones, and since the use of combinations of drugs is superior to single drug treatment [7] it was decided to try Prednimustine in combination with vincristine.

MATERIAL AND METHODS

AML group A was comprised of seven patients, six females and one male, aged 61–78 (mean 71) years. It was considered that chemotherapy was indicated when one or more of the following criteria were fulfilled: (1) anaemia requiring frequent transfusions, (2) granulocytopenia with infections, (3) thrombocytopenia with a tendency to bleeding. For remission induction Prednimustine was given orally in a daily dose of 20–60 mg. As maintenance treatment after the remission induction 16–20 mg of Prednimustine was given daily.

AML group B was comprised of four patients, two females and two males, aged 26–60

(mean 43). Treatment began immediately after the diagnosis and for remission induction 60–80 mg Prednimustine was given daily in three or four doses. In addition vincristine 2·5–3·5 mg was given i.v. once a week or once every two weeks. As maintenance treatment after remission induction, a daily dose of 24–30 mg of Prednimustine was given.

ALL. One patient was treated as the patients in AML group B.

Blood transfusions were given to all patients as required. Platelet concentrates were not administered. Infections were treated with various combinations of antibiotics without following any fixed schedule. Patients were considered to be in remission if their bone marrow contained no more than 5% of blast cells and if their blood granulocyte count and platelet count were at least 1500/μl and 100,000/μl respectively.

RESULTS

AML group A

In *two of the seven* patients complete remissions were obtained. The periods for remission induction are shown in Figs. 1 and 2. In patient E.P. remission was induced without a preceding pancytopenic phase. In patient K.G., with an unusually high neutrophil count at diagnosis, the number of neutrophils was just above 1000 μl on one occasion, seven weeks after starting treatment, but otherwise neutropenia or thrombocytopenia were not noted.

AML group B

Remissions were obtained in *two of the four* patients. Haematological data during the period of remission induction are given in Figs. 3 and 4. As in the elderly patients the remissions were not preceded by a drug-induced pancytopenia.

The period of survival after the diagnosis together with the time of survival after the start of chemotherapy, and the duration of remission for each AML patient are given in Table 1.

ALL. In the patient with ALL a remission was also obtained without preceding aggravation of neutropenia and thrombocytopenia (Fig. 5).

DISCUSSION

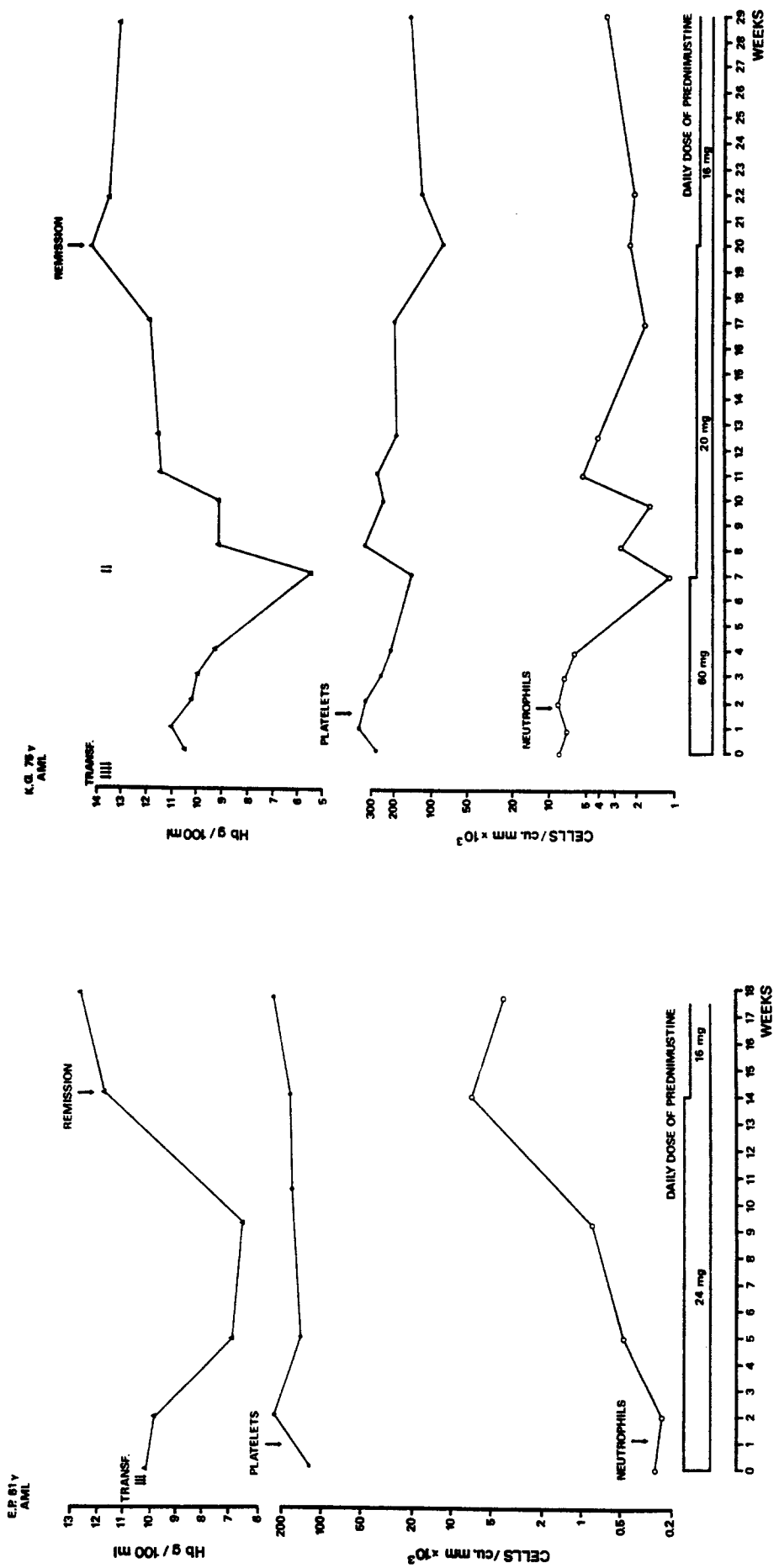
In this study of eleven adult patients with AML and one with ALL, five complete remissions were obtained, all without a preceding drug-induced pancytopenic phase. These data indicate that the current concept of bone marrow hypoplasia as a pre-requisite for remission in AML must be seriously questioned. It has been demonstrated that chemotherapy programs with only a moderate toxicity to normal haematopoietic tissue may be advantageous in adult AML since with aggressive therapy there is a considerable morbidity and mortality due to bone marrow depression during remission induction [16]. If a non-aggressive treatment results in a remission rate comparable to that obtained in patients treated more aggressively, it would be expected that the time of survival becomes lengthened in AML.

In our group of seven patients over the age of 60 two complete remissions have been obtained which may be comparable to the frequency of 15–30% remissions reported in larger series of aggressively treated elderly patients. In the younger adults three out of five have obtained remissions, two patients with AML and one with ALL. This result may be comparable to the frequency of 50–60% remissions reported in larger series of adult acute leukaemia [7].

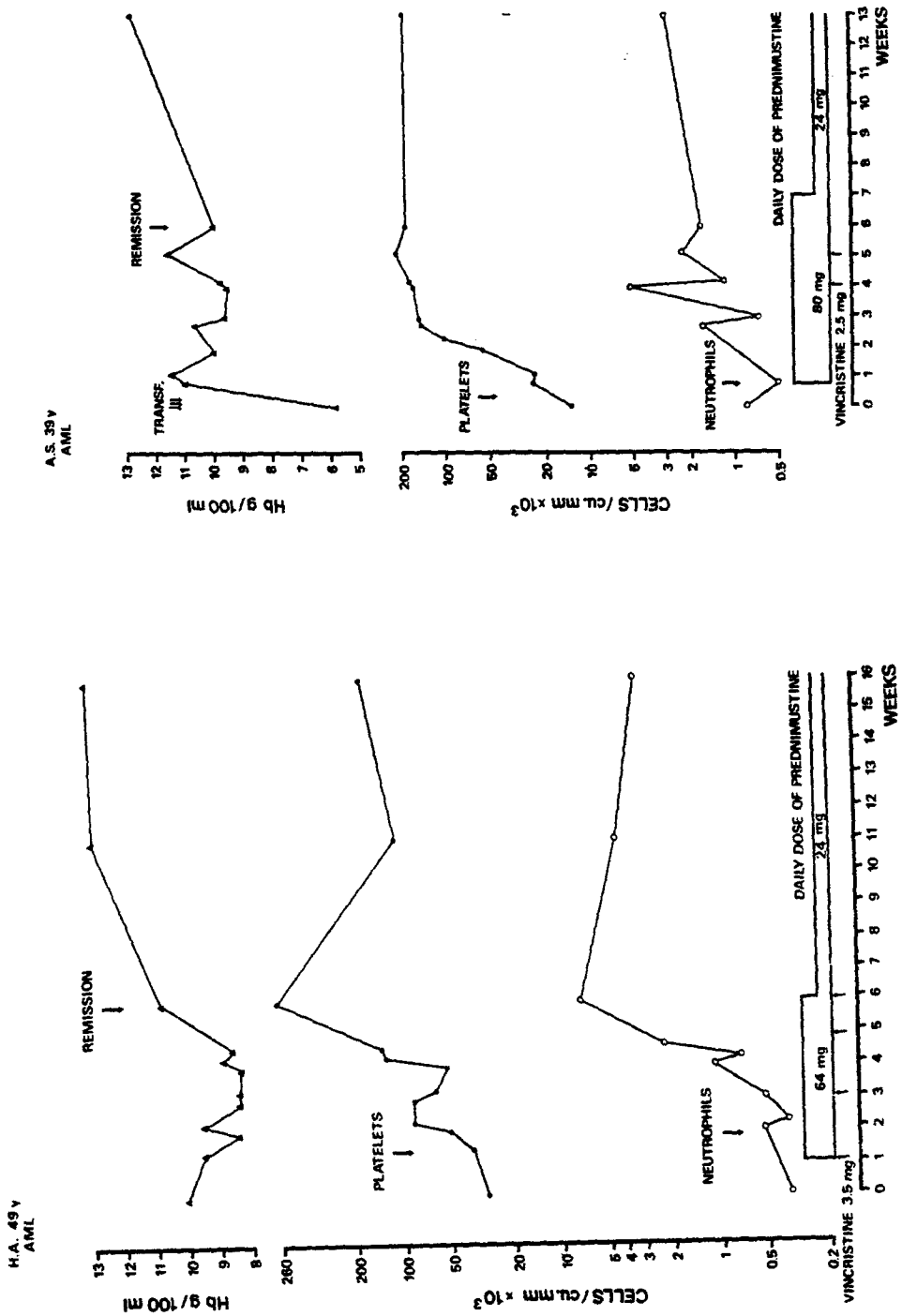
Obviously, more patients have to be studied before the remission rate, the periods in remission and the time of survival, can be compared to the results of more intensive treatment. However, nine out of our eleven AML patients (82%) have survived for more than six weeks after the start of chemotherapy.

Previous studies of aggressive treatment of patients with AML report a survival of more than six weeks in 67% [12], 52% [13] and less than 50% [11] of the cases. Although based upon a small number of patients with the high median age of 66, the results of this clinical study compare favourably with those mentioned above.

In cases of acute leukaemia, Prednimustine may reduce the amount of leukaemic tissue without seriously suppressing the normal production of blood cells within the bone marrow tissue. Despite the continuous administration of the drug, there was an increase in neutrophils and thrombocytes in several patients during remission induction. These findings are in good agreement with previous clinical studies [14, 15] regarding the low toxic effect of Prednimustine on normal bone marrow tissue, when patients with lymphocytic lymphoma and mammary carcinoma are exposed to comparable doses. The interesting possibility is indicated, that an alkylating agent may possess a more specific anti-tumour effect and a lower toxicity in normal cells, when bound to a corticosteroid.



Figs. 1–2. *Hematological data during remission induction in two elderly patients, 61 and 74 years old, with AML.*



Figs. 3–4. Hematological data during remission induction in two patients, 48 and 38 years old, with AML.

Table 1. Results of treatment with Prednimustine in AML

Group	Sex and age	Survival after diagnosis (months)	Survival after start of therapy (months)	Duration of remission (months)
A	F 78	11	10·5	—
	F 76	1	1	—
	F 76	1	0·75	—
	F 74	12*	10·5*	6†
	F 68	4*	4*	—
	M 66	14	1·75	—
	F 61	12·5	11	9
B	M 60	4·5	4·5	—
	F 48	21*	21*	20†
	M 38	5*	5*	4†
	F 26	2	2	—

*Still surviving.
†Still in remission.

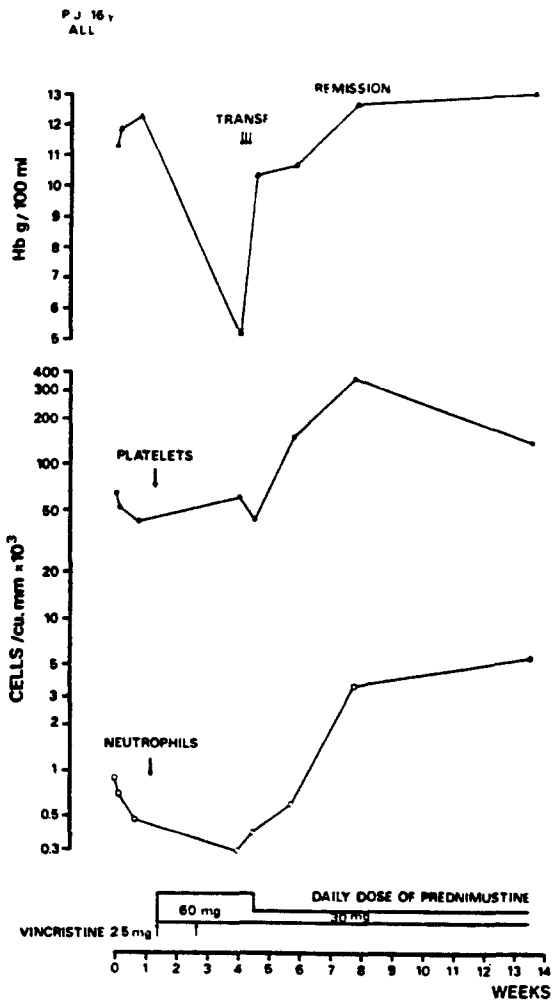


Fig. 5. Hematological data during remission induction in a 16 years old male with ALL.

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Induction Chemotherapy of Non-Hodgkin's Malignant Lymphomas

Preliminary results of a controlled trial*

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Abstract—*Thirty-three patients have been included in a randomized trial comparing cyclophosphamide + vincristine + prednisone (CVP) + VM 26 (CVPV) and CVP + adriamycine (CVPA). The tolerance is good for the two regimens. There is no difference between the two protocols, which clearly give better results than CVP alone.*

NON-HODGKIN'S malignant lymphomas are very often disseminated and this requires a systemic treatment by chemotherapy. After two previous controlled trials comparing several modalities of a triple association with cyclophosphamide, vincristine and prednisone (CVP, 1, 2), we have studied the usefulness of adding VM26 or adriamycin to CVP. Preliminary results of this new trial are reported here.

MATERIAL AND METHODS

From 1st April, 1974 to 30th October, 1975, 33 patients were included. For all patients the diagnosis of non-Hodgkin's lymphomas was verified cytologically and histologically and there was an indication of induction chemotherapy before further radiotherapy or maintenance chemotherapy. No patient over 70 years old was included. These patients were assigned randomly to one of the two regimens of chemotherapy. In spite of the randomization, the prognostic factors were significantly worse for the patients of the first group than for those of the other one; the main characteristics are shown in Table 1.

The two regimens of chemotherapy are detailed in Table 2. One cycle only was given to each patient. All applied treatments are evaluable.

Table 1. Main characteristics of patients included in the trial

		Protocols	
		CVPV	CVPA
Male/Female		14/4	13/2
Age (mean; range)		51·6 (17–68)	55·4 (42–68)
Histological type	diffuse	15	14
	nodular	3	1
Perceptible phase	No. 1	6	11
	No. 2	10	4
	No. 3	2	0
Previous treatment	Radiotherapy	10	13
	Chemotherapy	8	2
Clinical Stage	I	5	6
	II	2	2
	III	4	6
	IV	7	1

RESULTS

On the whole the tolerance was excellent. No treatment was stopped because of clinical toxicity; 3 treatments with CVPV and one with CVPA were stopped because of hematological toxicity.

The efficiency of treatment, as evaluated immediately after the end of chemotherapy is shown in Table 3. It appears the complete failures were obtained only with CVPV but it seems that this fact is related mainly to osseous

*Communication to the First Congress of the Medical Oncology Society, Nice, December 2, 1975.
†Presented by B. Hoerni.

Table 2. Protocols of chemotherapy

		CVPV	CVPA
Cyclophosphamide	400 mg/m²	day 3, 10 and 17	day 1, 8 and 15
Vincristine	0, 7/m²	day 1, 8 and 15	day 1, 8 and 15
Prednisone	40 mg/m²	days 1–15	days 1–15
VM 26	70 mg/m²	day 5, 12 and 19	—
Adriamycine	35 mg/m²	—	day 1 and 15

Table 3. Tumour regression

	CVPV	CVPA
Number of patients	18	15
Complete failure	4	0
Tumour regression < 50%	2	3
Tumour regression > 50%	7	5
Complete regression	5	7

Table 4. Historical comparison between CVP alone and quadruple chemotherapy

	CVP*		CVPV+CVPA	
Number of patients included	90		33	
Complete failure	5		4	
Tumour regression <50%	38		5	
Tumour regression >50%	43	52%	12	73%
Complete regression	4		12	

*Previous personal trials.

localizations treated with this protocol. On the whole there is no significant difference between the two regimens.

COMMENTS

Until now there has been no difference between the effects of the two regimens of chemotherapy studied in this trial. Nevertheless, it is possible to make an historical comparison with previous trials (Table 4). It appears that the addition of VM26 or adriamycine to CVP offers a significant improvement in comparison with CVP alone for complete regression, or all regressions of more than 50%. The toxicity is mild but no patient over 70 years was treated.

This trial is going on currently, and the place of these induction chemotherapeutic regimens in the over-all treatment of non-Hodgkin's lymphomas is now under study.

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Remission Induction Chemotherapy in Acute Myeloid Leukemia

Preliminary results

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Abstract—*In 7 cases of proliferative AML, a pre-induction of 5 days of L-asparaginase followed by a chemical induction combining daunorubicine and cytosine arabinoside, has allowed us to obtain 6 complete remissions out of which 5 were durable. This preinduction has shown a very marked decrease of blood cells in 5 cases out of 7.*

INTRODUCTION

INDUCTION treatment of acute myeloid leukemia has made great progress in the last decade, producing in most centres having hematologic support facilities, more than 50% complete remissions during the first phase of the illness.

The application of a protocol combining:

(1) cytosine arabinoside: 100 mg/m² in 8 hr infusion every day, from day 1 to day 7, and

(2) daunorubicine: 60 mg/m² i.v. day 1 and day 2, has allowed us to obtain 60% complete remissions in 20 cases treated with a mean remission of 10·5 months.

The analysis of failures of this treatment shows the importance of age, the frequency of fatal accidents during induction and the resistance of proliferative tumour types with hepatosplenomegaly. For this last type, we have modified our protocol: the increase of dosage did not increase the efficacy and resulted in the increased frequency of lasting and fatal aplasia. The addition of another drug was necessary and we chose L-asparaginase despite the studies against its efficiency in AML [1]. The use of L-asparaginase alone would give only 10% complete remission [2]. However, in most cases, this treatment shows an important drop in circulating myeloblasts [3].

A few new trials have shown good results in chemotherapy combinations using L-asparaginase [4] and have convinced us to make this choice.

L-Asparaginase administered in pre-induction allows a great reduction of myeloblasts without important modification of the normal cells.

MATERIAL AND METHODS

We have used as a scheme:

L-asparaginase: 50,000 U/d i.m. or i.v. d1 and d5,

daunorubicine: 60 mg/m²/d i.v. d6 to d7,

cytosine arabinoside: 100 mg/m²/d in 8 hr infusion d6 to d7.

This treatment was used on 7 patients with a proliferative acute myeloid leukemia. The patients' age varied between 15 and 62; 6 patients were at the first phase of their disease, one at the first relapse.

RESULTS

We have observed one death during induction due to a hemorragic syndrome. Six treatments have succeeded without intercurrent incident. Aplasia was constant and durable in all cases. This aplasia lasted from 18 to 31 days (mean: 24 days) during which time white blood cell transfusions were necessary.

Complete remission was obtained in 6 cases where we applied the treatment completely. In 5 cases this remission was excellent and actually these observations have been noticed for a period of 4 months. In one case, one patient in remission died two months after the treatment from a cardiac accident. In one case of complete remission we noted a first relapse.

The analysis of these remissions showed that, out of 7 cases 5 had a drop of peripheral white

blood cells during preinduction with L-asparaginase, bringing the patients to 3/5000 leucocytes during induction.

This treatment has also been used in 2 cases of transformed CML resulting only in 2 partial remissions.

DISCUSSION

These results led us to simplify our therapeutic protocol:

in the initially low proliferative types, in elderly patients, we use the 2 drug induction combination especially when we cannot count on prolonged hematologic support

in proliferative types, when donors of white blood cells are available, the triple-drug combinations can be used.

This approach can be extended later if the quality and the duration of the remission obtained by this means proves itself superior to the classic treatment.

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Treatment of Adult Acute Myeloid Leukemia

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Abstract—*Between November 1974 and October 1975, 50 acute myeloid leukemias in first perceptible phase received according to their initial stade, an induction treatment either with daunorubicine vincristine cytosine arabinoside or with cyclophosphamide, vincristine, cytosine arabinoside in reduced dose.*

When, in complete remission, patients were treated with consolidation and maintenance cyclic chemotherapy, prevention of central nervous system involvement, androgenotherapy and, by randomisation immunotherapy or not with B.C.G.

An overall remission rate of 68% and 75% with daunorubicine induction regimen were obtained; it is too early for the evaluation of remission duration.

THE TREATMENT of adult acute myeloid leukemia has been significantly improved during the last few years. Combination chemotherapy, improvement in supportive care, immunotherapy or other stimulating factors are responsible for better therapeutic efficiency [1–3]. We report here the preliminary results of a protocol (L 275) for the induction and prolongation of remission in acute myeloid leukemia.

1. PATIENTS AND METHODS

(1) *Patients*

Between November 1974 and October 1975, 50 new patients entered in our center with A.M.L. in first perceptible phase. All these patients were included. There were 27 males and 23 females whose age ranged from 15 to 78. Ten patients were over 65, of these 8 were females, and median age was 72. Other 40 patients were under 65 with a median age of 50.

In all patients, bone marrow smears were classified on the basis of the subdivision of acute leukemias proposed by Mathe *et al.* [4]. 43 were typical myeloblastic leukemias, 5 promyelocytic leukemias, 3 myelomonocytic leukemias.

Peripheral white blood cells count varied widely as shown in the Table 1 and tumoral involvement was noted in 14 patients.

(2) *Treatment protocol*

(2.1) *Remission induction.* Induction treatment was modulated according to the initial state of patients and therapeutic response. Two inductions regimens were used (Table 2):

Either, three drugs: daunorubicine (DNR), vincristine (VCR) and cytosine arabinoside (CAR) were administered in a seven days course which may be prolonged or shortened according to the peripheral white blood cells count.

Or in patients over 65 and/or with cardiac, hepatic or renal disease, thus presenting a high risk of treatment failure, a five days course was

Table 1. *White blood cell count at the onset of treatment*

W.B.C. × 10^3	0	10	50	150
Patients	20	12	18	

Table 2. *Induction remission schedule (dose in mg body surface area)*

Days	1	2	3	4	5	6	7
"Normal" course							
Daunorubicine	60	60					60
Vincristine			1				
Cytosine arabinoside				240	240	240	240
"High risk" course.							
Cyclophosphamide	600						
Vincristine		1					
Cytosine arabinoside			210	210	210	210	210

Modulation in normal course: Day 7 if WBC $\geq 1500/mm^3$. Cytosine is given on day 8 WBC $\leq 1000/mm^3$. No Daunorubicine injection on day 7.

performed where cyclophosphamide (CPM), took the place of daunorubicine and cytosine dose was reduced.

During the induction phase, all available supportive care was used: isolation, quick metabolic-defect correction, platelet or white blood cells given if necessary in large amounts, empirical and massive antibiotherapy once fever occurred.

(2.2) *Maintenance therapy.* When patients achieved complete remission (C.R.) three successive phases were set up in the treatment.

(*a*) Consolidation therapy. A new course of induction regimen was given once per month for three months. In normal regimen the last daunorubicine injection was omitted, so that the total daunorubicine dosage at the completion of the consolidation was 540 mg/m^2.

(*b*) Prevention of central nervous system involvement. After a period of 2 weeks rest, 2400 rads were delivered in 18 days over the skull above C2. During the same period of time 5 injections of 12 mg/m^2 of methotrexate were given intrathecally.

(*c*) Maintenance therapy. After another 2 weeks rest, an intermittent five days course of chemotherapy (Table 3) was instaured on every 4 weeks during the first three months, then on every six weeks for three months and then systematically every eight weeks.

Table 3. *Maintenance chemotherapy course dose mg/ b.s.a.*

Day	1	2	3	4	5
Cyclophosphamide	600				
Methylglyoxal	200				
Vincristine		1			
Cytosine arabinoside			210	210	210

After the first course of consolidation therapy a randomisation was made between chemotherapy and chemotherapy plus immunotherapy. In the latter case, Institut Pasteur BCG was applied by means of a heaf gun: 4 multiple punctures at each time, at weekly intervals, and was stopped 2 weeks before the next chemotherapy course.

All patients received also after the first consolidation course, oral androgenotherapy with stanozolol (2 mg/kg).

2. RESULTS

(1) *Remission induction.*

Complete remission was obtained in 34 cases. Failure and death during induction occurred in 16 cases. With the "normal" regimen given to the 40 patients, 30 (75%) were in remission. With the "high risk" regimen, 4 patients out of 10 were in remission (40%). The overall remission rate for all patients was 68%.

Table 4. *Results of induction treatment*

Protocol	Normal	High risk	Total
Total	40	10	50
C.R.	30	4	34
%	75	40	68
Died in induction	10	6	16

In the normal regimen, the remission arises between the fifteenth and the twenty-fifth day, with a median at eighteen days. Thirty patients entered in remission: 27 after one course, and in three cases, complete remission was only achieved after a second induction course. In 25 cases, a complete course was realised, in 7 cases another day of cytosine was administered and in 8 cases, the course was shortened with no daunorubicine injection on the seventh day.

In the regimen for patients with a high risk of failure, 4 complete remissions were obtained after 2 and 3 courses of treatment.

Life threatening complications occurred frequently during the induction phase. Infectious complications with fever for more than five days were almost constant and were the major risk. Hemorrhage due to thrombocytopenia or disseminated intravascular coagulation present in 6 cases were also dangerous.

(2) *Complete remission.*

It is too early for the evaluation of remission duration and there is no sufficient patient to evaluate immunotherapy and androgenotherapy.

Seven patients died, three of which were older than 65. Five patients relapsed and two others died from intercurrent disease in complete remission.

Table 5 shows the duration state of 34 patients in C.R.

Toxicity or side effects of treatment of remission were not limiting. Headaches are frequent during skull irradiation and after lumbar punctures. Alopecia is common.

3. DISCUSSION

Improvement in remission rate of acute myeloid leukemia arises by two ways:

(1) Intensive combination chemotherapy re-

Table 5. *Survival from diagnosis* 34 *patients in C.R.*

Alive		+		+	+				+			
		+		+	+		+	+	+			
		+	+	+	+		+		+			+
In C.R.		+	+	+	+	+	+	+	+	+	+	+
Month	1	2	3	4	5	6	7	8	9	10	11	12
Relapsed					±			±				
and			±			±	±					
died					±			±				

duces quickly the total body burden of leukemic cells and so minimises the duration of aplasia before occurrence of remission [5].

(2) Better and more effective prevention and supportive care overcome more frequently live threatening complications [6]; with such a management in induction, remission rates of 50 percent or more become the rule.

McCredie [7] with adriamycine, vincristine, cytosine arabinoside reports a 83% remission rate in 46 patients. Yates, [8] with cytosine and daunorubicine obtains 87% remission rate, in patients under 60 years old and 25% in patients over 65. Holland [9] shows that seven and three days course of cytosine arabinoside and daunorubicine are better than five and two days course to inducing remission.

In this study, similar results appears with an overall remission rate of 68 and 75% with the normal induction regimen.

Now, improvement in the duration of the remission must be obtained: cyclic chemotherapeutic course, immunotherapy with BCG or extracts, blast cells, or other stimulating factors, like androgenotherapy seem useful and promising and must be investigated [10].

The results of the L 275 protocol to date appear promising but the brevity of the study precludes any prediction at the time, as to the eventual duration of complete remission.

Obtention of C.R. rate as in ALL with less complications during induction, and exploration of the best regimen remission are now the major step in the treatment of AML.

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Kinetic Conditions Preventing the Eradication of Human Leukemia*

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Abstract—*Attempts to exploit the proliferation kinetics of human leukemic cells in order to improve therapeutic protocols have been made in recent years. While in some instances it has proved possible to eradicate the disease in animals, relapse is a virtually constant feature in humans.*

Failure in this respect can be explained in terms of kinetic data themselves, keeping in mind that there are at least three main kinetic obstacles to the definitive eradication of human leukemia:

— *the presence of a large number of cells that are out of cycle and hence less susceptible to the drug attack;*
— *the persistence, even after treatment, of cells in a Go position with a potentiality of leukemic stem cells;*
— *the persistence of a contrary flux from Proliferating to Go compartment even during the action of drugs.*

INTRODUCTION

THE MANAGEMENT of human leukemia has been improved mainly by the analysis of the proliferation pattern of leukemic cell populations besides the introduction of polychemotherapeutic associations. The fact remains, however, that remissions occur in 90% of lymphoblastic and 50% of myeloblastic forms and, what is worse, relapses are particularly frequent, so that cases of real cure are quite exceptional.

Kinetic studies, even if proved so far incapable of giving a definitive support to a radical treatment of the disease, are prepared to give explanation of their own lack of success that deserves consideration. An analysis will be made, in this paper, of the kinetic conditions present in human acute leukemia, which can be considered the greatest obstacle to the eradication of leukemic cell population with present drugs.

MATERIAL AND METHODS

The results of kinetic investigations on human acute leukemia developed over the last fifteen years in this and other laboratories are considered for the present study. As a conclusion of these investigations a kinetic growth model has been proposed for acute leukemia cell population: it includes a Go phase blast pool, whereas the leukemic stem fraction is considered as consisting of part of the blast population, the majority of the fraction itself being out of cycle (Go) at any given moment: one quota only of stem cells is active and feed the proliferating compartment which, by itself, is not selfmaintaining [1, 2].

ESULTS AND DISCUSSION

The pool of leukemic blast cells out of cycle (Go) that are relatively insensitive to the drug attack and, since they can act as stem cells, able to insure continuous replenishment of the proliferating compartment, is the first main obstacle to the eradication of the acute leukemia in man [3–5]. Only a part of leukemic cells are, in fact, responsive to cycle and phase specific drugs, while non cycle specific drugs have a poor effect on non proliferating blasts at the dose we must employ them. Consequently a more effective killing of leukemic cells cannot be achieved with present drugs except by complete recall into the cycle of blasts that become quiescent during the growth of the leukemic population. It is now clear that even this objective is out of reach and growth fractions (G.F.) near unity have never been attained in acute leukemia by procedures, such as synchronisation or recruitment, performed in

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order to increase the quota of cycling blasts.

The impossibility of eradicating all blasts in Go is particularly due to the following reasons:

(i) Asynchronous flux of blasts from Go to cycle after recruitment induced by drugs. In fact, the flow from Go into proliferating compartment is always discontinuous, even when it is augmented by drugs. Furthermore, it is always incomplete: the highest labelling index values so far obtained after administration of appropriate drugs are always associated with G.F. increases lower than unity [6]. This means that a Go compartment still persists with the presence of a true pool of leukemic stem cells capable of insuring self-maintenance and re-expansion of the blast population.

(ii) Persistence of a contrary flux from proliferating to Go compartment even during treatment. This fact, already suggested as a hypothetical possibility [6], has been recently proven by Pagliardi *et al.* [7] by studying two cases of acute lymphoblastic leukemia during the action of high doses of methotrexate. Both cases were also injected *in vivo* with tritiated thymidine. The label was given along with the drug in one case and just after its administration in the other. The persistence of a flux from proliferating to Go pool has been proven by the following evidences: blasts, labelled with the pulse of tritiated thymidine, persisted as non proliferating cells, as long as it was possible to detect leukemic cells in the peripheral blood after the administration of the drug; labelled mitoses appeared some days after the administration of the methotrexate: evaluation of DNA content showed that these mitoses gave rise to a population of non proliferating cells behaved as Go cells since they recommenced a proliferating activity after a long period of mitotic rest.

The fact that the existing drugs fail to discriminate between leukemic and normal stem cells is another main obstacle to the eradication of the leukemic cell population.

A marked failure of normal haemotopoiesis is a distinctive feature of leukemia. After remission induced by chemotherapy, however, the persisting normal stem cells are able to repopulate the bone marrow and release almost normally functioning blood cells. This situation can now be maintained for a long time with an appropriate administration of certain drugs, but eventually the leukemic cells return in detectable number and relapse will occur. Knowledge of the source of the cells which repopulate the bone marrow in remission and of the leukemic stem cells which reappear in relapse is scanty. It is probable that, when a high percentage of both leukemic and normal stem cells are destroyed by the therapy, the difference in proliferative speed, migrating and colonizing ability, will favour the return of normal haemopoiesis. In fact, as well known, in leukemia the malignant cells divide more slowly than normal ones. Furthermore, the results of the *in vitro* colony growth technique applied to human leukemic bone marrow revealed the paucity of colony forming cells in relapse and an almost normal number in remission [8, 9]. It is apparent that the activity of normal stem cells is suppressed when leukemic cell population develops. The understanding of the mechanisms leading to the suppression of normal stem cell activity in the course of leukemia is thus of cardinal importance: at present, however, we do not even know whether normal stem cells are reduced in number or merely inhibited in their ability of feeding the normal blood cell population.

We must consider that, when we start with a chemotherapy, we are dealing with a kinetic situation in which exists a marked failure of normal haematopoiesis, whatever this may be. Chemotherapy leads to a considerable reduction of the leukemic cell mass and hence of the leukemic stem cells: however, a persistent activity of leukemic stem cells even during the so-called "complete" remissions, is an almost constant feature [10]. This means that a sufficient number of leukemic stem cells able to insure the re-expansion of the leukemic cell mass have survived the action of the drugs, even if these are given at dosages near to the toxicity, and that remission is mostly based on the kinetic differences between normal and leukemic cells. Consequently, if we want to increase the reduction of leukemic cell population with present drugs incapable to display a discriminative effect between normal and leukemic cells, we have to push the treatment to a limit in which a severe bone marrow asplasia supervenes with the disappearance of the normal stem cells too.

CONCLUSION

From the above kinetic analysis the following conclusions can be drawn: (1) a complete eradication of a leukemic cell population requires the destruction of all its clonogenic cells; (2) the poor discrimination between normal and leukemic stem cells displayed by the drugs now available means that a complete eradication cannot be achieved without destroying at the same time the normal stem cells.

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Combination Chemotherapy with Adriamycine, VM 26, Cyclophosphamide and Prednisone in Lymphosarcoma and Reticulosarcoma Stage III and IV

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Abstract—*This work presents the results obtained on 34 patients with disseminated lymphosarcoma and reticulosarcoma (Stage III and IV) with a cyclic combination of chemotherapy which combines adriamycin, epipodophyllotoxine (VM 26), cyclophosphamide, and prednisone. The complete remission rate is 56% of the patients who entered the trial, the response rate is 85%. Tolerance of the regimen is good. The study also demonstrated the prognostic value of the new histocytological classification of lymphosarcomas proposed by W.H.O.*

INTRODUCTION

THERAPEUTIC management of lymphosarcoma and reticulosarcoma has been modified in recent years under the influence of (a) better knowledge of the disease which has shown that most cases are already disseminated at the time of diagnosis [1–3] and are therefore, to be treated by chemotherapy, (b) discovery of new drugs which proved efficient in the treatment of these tumors, particularly VM 26 or dimethyl epipodophyllotoxin [1, 4] and adriamycin [5–7].

We present a preliminary study of a new combination of adriamycin, VM 26, cyclophosphamide and prednisone in the treatment of disseminated lymphosarcoma and reticulosarcoma, stage III and IV. This combination is based on the activity of each drug used alone [1, 4–8] and on the synchronization and potentiation obtained by sequential administration of adriamycine, VM 26, cyclophosphamide [9, 10].

It must be emphasized that some patients in the trial had already been treated by other chemotherapeutic regimens and/or radiotherapy. These patients can only make the results appear worse as compared to a population of patients in the first perceptible phase of the disease.

PATIENTS AND METHODS

Thirty-four patients (22 males and 12 females) aged 5 to 67 who received AVmCP regimen, as described in Table 2. All had lymphosarcoma or reticulosarcoma stage III or IV.

Twenty-one percent of the patients were in the first perceptible phase of the disease; 11 had relapsed once, and two were in the third perceptible phase.

The patients were classified according to the new histological classification of lymphosarcomas and reticulosarcomas proposed by W.H.O. [11].

Seven patients had "immunoblastic lymphosarcoma" stage III and IV; 4 had "lymphoblastic lymphosarcoma", 6 had "prolymphocytic lymphosarcoma", among which were the two "nodular lymphosarcoma" of the series; 5 had reticulosarcoma, two of which were bone primary localisation with lymph-node or pulmonary matastases. 12 patients could not be classified because of the lack of cytologic examination of the lymph nodes.

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Table 1. *Treatment of lymphosarcoma and reticulosarcoma stage III and IV by AVMCP results*

	C.R.	I.R. 50%	Failure	Total
Immunoblastic	2	3	3	7
Lymphoblastic	4	0	0	4
Prolymphocytic	4	2	0	6
Reticulosarcoma	3	2	0	5
Unclassified	6	3	3	12
Total	16 56%	10 29%	5 15%	34

Table 2. Protocol of treatment of lymphosarcoma and reticulosarcoma stages III and IV with adriamycine, VM 26, cyclophosphamide and prednisone

Day 1	Adriamycine	40 mg/m²	I.V.
Day 2	VM 26 (epipodophyllotoxin)	60 mg/m²	I.V.
Day 3–4	Cyclophosphamide	300 mg/m²	I.M.
Day 3–4–5–6–7	Prednisone	40 mg/m²	P.O.

Table 3. Toxicity

Leucopenia 1·500 WBC	14
Thrombocytopenia 50·000	4
Regressive infection	2
Lethal infection	0
Alopecia	29
Nausea	2
Heart failure	1
Asthenia	0
Severe diabetes	1
Haemorrhagic cystitis	1
Pseudo anaphylactic acute intolerance to VM 26	1
Renal failure	2

Chemotherapeutic regimen is described in Table 2. Each cycle combines, on day I i.v. injection of adriamycine 40 mg/m², on day 2 VM 26 60 mg/m² by intraveinous infusions, on day 3 and 4, cyclophosphamide 300 mg/m² i.m. or i.v., on days 3–7, prednisone given orally at the dose of 40 mg/m².

The interval between two cycles, necessary to hematologic and immunologic restoration is 15–21 days. Eight such cycles are given in about six months.

(1) *Toxicity*

Toxicity is described in Table 3. It is mild or moderate. Leukopenia under 1500 white blood cells was observed in 14 cases with only two cases of regressive infection. Thrombocytopenia under 50,000 platelets occurred in 4 cases. No hemorrhagic complication was observed. Alopecia was practically constant. Nausea was mild. Other complications were rare.

(2) *Anti-tumor effect*

The overall response rate is very high. 85%, with a complete remission rate of 56%. Although the number of patients is still too small to draw definite conclusions, it seems that the various histocytological types have different sensitivity to the regimen. Two patients with immunoblastic lymphosarcoma failed to respond to the regimen, while the four patients with lymphoblastic lymphosarcoma and the four patients with prolymphocytic lymphosarcoma in the first perceptible phase underwent complete remission.

Moreover when complete remission was achieved it never lasted more than six months in immunoblastic lymphosarcoma while the duration of remission was over one year in all patients but one with lymphoblastic or prolymhocytic lymphosarcoma.

The remissions were obtained very rapidly, or often after the first cycle and all patients who underwent complete remission had achieved t after three cycles.

Figures 1 and 2 are tentative survival curves of all patients in the first perceptible phase

(Fig. 1) and of patients who underwent complete remission (Fig. 2). They seem to us very encouraging although they cannot yet be considered as significant.

DISCUSSION

We present a therapeutic regimen which shows a very high degree of efficacy in lymphosarcoma and reticulosarcoma. The drugs were chosen because of their maximal efficacy for minimal toxicity. Drugs with the high degree of toxicity like nitrosoureas were excluded from the regimen and tolerance of the regimen was remarkably good, at least on patients who had not been previously irradiated.

Although further study has to be done with a greater number of patients and longer time of observation, it seems that the classification proposed by W.H.O. for lymphosarcoma is of significant prognostic value.

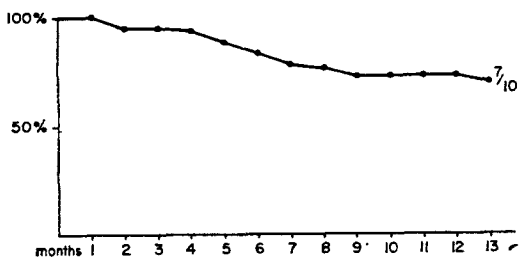

Fig. 1. Survival curve of 21 patients with lymphosarcoma and reticulum cell sarcoma. Stage III and IV treated with AVmCP in first perceptible phase.

Fig. 2. Survival curve of patients in C.R. with lymphosarcoma and reticulo-sarcoma. Stage III and IV treated with ADM, VM 26, CPM and PDN (N = 19 patients).

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Cure of Metastatic Tumors by Chemotherapy

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Abstract—*Twelve patients presenting metastatic tumors of various origin (testicular, ovary, prostate, uterus, soft part) have been treated more than five years ago by chemotherapy alone. An association of drugs (actinomycin D, cytoxan and methotrexate) or a monochemotherapy (either nitromin, trenimon, thiotepa or alkeran) have been used.*

The twelve patients are alive and well from 15 to 5 yr after.

In view of these good results we have treated all dysembryoplastic testicular tumors with chemotherapy since 1962. We add chemotherapy after surgery (orchidectomy and lymphadenectomy) whether the nodes are involved or not.

All of 17 patients without involved nodes are alive and well more than 3 yr and 13 out of 20 with nodes involved.

1. INTRODUCTION

IT WAS not previously thought possible to cure patients with metastatic disease by chemotherapy alone. Mathé and Kenis in a recent book and Brulé *et al.* state that only uterine choriocarcinoma and Burkitt's lymphoma are cured by chemotherapy alone. Perhaps some children too (such as those with neuroblastoma and sometimes Wilms tumors) can be cured.

Since the Houston Congress on Cancer a number of cases of apparent cures by chemotherapy have been published. We think that a complete regression of metastatic tumor for at least 5 years is a cure.

2. MATERIAL AND METHODS

Twelve patients having metastatic disease from various primary tumors were treated more than 5 years ago by chemotherapy, they are alive and well without evidence of disease.

Report on the first 6 cases was published in the Journal of Surgical Oncology in 1974 [3].

Cases no. 1, 3 and 4 are patients with dysembryoplastic testicular tumors with lung metastases. They were treated by the association of actinomycin D, cytoxan and methotrexate or nitromin. They are alive and well 10, 12 and 15 years afterwards.

Case no. 2 was also a patient with dysembryoplastic testicular tumor with lymph nodes involved and skin metastases. All disappeared with chemotherapy 11 years ago and there has been no recurrence.

Case no. 5 presented recurrent metastatic lymph nodes in the neck after surgery for a trabeculo-vesicular thyroid cancer. All nodes disappeared within two months of chemotherapy. He is alive and well 10 years later.

Case no. 6 was a young man presenting a small-cell sarcoma of the prostate with lung metastases which disappeared after chemotherapy. He is still well 14 years later.

Case no. 7 is a 37 year-old man presenting a large pelvic mass which had been incompletely removed (a mass of 4 cm dia was left. It was a malignant mesenchymoma). He then received a course of actinomycin D, cytoxan and methotrexate every 3 months for 18 months and was operated 19 months after the first surgical treatment because of persistent pain; there remained nothing in front of the sacrum and the histologic biopsy revealed only fibrous tissue.

He received one more course of chemotherapy after surgery and he is still alive and well without evidence of disease 5 years later.

Case no. 8 is a young woman operated on at the age of 10 for a benign right ovary tumor (folliculome). Five years later during an appendicectomy all was found well in her abdomen; 3 years later (she was 18 years old) she had a large tumor in her right side removed, it was a malignant dysembryoplastic tumor.

She had then one pregnancy, delivering a normal child and remained well for 11 years.

When 27 years old she felt pain in her right side and a large mass under her liver was discovered 2 years later. At this time there were lung metastases and a large calcified mass squeezing the right kidney. She received 2 courses of cytoxan, methotrexate and rufocromomycin. All lung metastases disappeared. Then she received actinomycin D, cytoxan and methotrexate and her I.V.P. was normal again. She was operated upon and a mass of 8 cm in diameter was removed, histologically it was necrotic tissue only. She is still alive and well, 7 years later.

Case no. 9: this 39 year-old woman had tumors of both ovaries removed, she had metastatic nodules everywhere in her abdomen even on the liver. She received some cobalt irradiation on her pelvis and a hormonal treatment (i.e. norfor* 80 mg/day for 4 years) and chemotherapy with thiopeta every week for one year, then every two weeks for one year. A further operation for an eventration 4 and a half years later revealed no lesion.

Case no. 10: this 47 year-old patient had bilateral ovarian tumor with ascites and lesions involving the diaphragm. She received first cytoxan without success, then alkeran every two months for a year, then every 6 months (2 courses). She is alive and well 5 years later without evidence of disease.

Case no. 11: this 47 year-old patient had had an hysterectomy for an uterine leiomyosarcoma. At that time she had mediastinal metastatic lymph nodes. She received rufocromomycin, cytoxan and methotrexate every 3 months for a year. All lesions disappeared, she is alive and well 5 years later.

Case no. 12: this 49 year-old patient had an hysterectomy in 1963 for a fibrom. Five years later she was operated upon for large and numerous tumors all over her abdomen. They were classified as "sarcome du chorion cytogene". She then received cytoxan and methotrexate a weekly dose two weeks running each months over a period of 6 months then every two months over a period of 18 months. She is still alive and well 7 years later.

We have left out the cases of uterine choriocarcinoma and those of neuroblastomas which are nowadays easily cured in a certain number of cases, by chemotherapy. We have left out the cases without histologic proof or without distant metastasis even if cured by chemotherapy alone.

*Norethindrone.

3. RESULTS

Twelve patients presenting either lung or lymph nodes metastasis or local recurrence from a testicular tumor in 4 cases, an ovarian tumor in 3 cases and uterine sarcoma in 2 cases and a soft part sarcoma, small cell sarcoma of the prostate and thyroid cancer have been treated with courses of a combination of drugs mostly actinomycin, cytoxan, methotrexate or monochemotherapy.

All their metastases have disappeared and they are alive and well without evidence of disease more than 5 years after.

4. DISCUSSION

All our patients were treated more than 5 years ago. Recurrences may happen 3 years after complete regression of metastatic lesions as Rutledge and we ourselves know such as in ovarian or testicular tumors. Five years is perhaps not long enough but so far we have not seen any recurrence after 5 years, unlike Chabner.

Those 12 patients had different primaries but there is an histologic pattern, only some tumors can regress by chemotherapy alone such as: testicular malignant dysembryoma, soft part sarcoma, adenocarcinoma of the ovary or cancer of the thyroid.

We have not seen cures in melanomas or kidney tumors which have been known to regress spontaneously.

The doubling time of the metastases in the lung had a very wide range of time. In patient no. 3, one metastasis had a doubling time of 6 days and in the same lung another metastasis had a doubling time of 21 days. In some cases the doubling time was very long as in ovarian tumors.

Our patients were rather young, the youngest being 14 years old, the oldest 61 years old, the mean age was 34 years.

Not every metastasis can regress. Here only lung, lymph nodes, intra abdominal nodules or skin nodules regressed. The type of chemotherapy was variable, some patients received only one drug, others had an association of 3 drugs, as done by Li; in 2 cases actinomycin D was replaced by rufocromomycin. As a rule the treatment lasted 2 years but in some cases it was stopped after one year and in 2 cases was pursued 4 or 5 years.

In a case the regression was apparently complete after the first course of treatment.

In some rare cases if we can cure patients with large metastatic tumors, radiologically visible how much more should we be able to

cure lesions only microscopically detectable. Studies on cellular kinetic and tumor volume seem to confirm this; so the fact that some patients can be cured by chemotherapy only is a positive argument for treating patients with adjuvant chemotherapy after surgery so as to try and clean out any metastasis which might be present even if not visible.

We add chemotherapy after surgery (orchidectomy and lymphadenectomy) in all dysembryoplastic testicular tumors whether the lymph nodes are involved or not.

With no nodes microscopically involved all of 17 patients treated more than 3 years ago are well with no evidence of disease and 13 out of 20 with nodes involved.

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Treatment of Malignant Gliomas in Adults Using a Combination of Adriamycine, VM 26 and CCNU: Results of a Type II Trial

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Abstract—*Forty-three patients with inoperable and/or recurring malignant gliomas were treated with a combination of Adriamycine (45 mg/m²) and 4-dimethyl-epipodophyllotoxin D-thenylidene (VM 26) (60 mg/m² for two days). These cycles of treatment were repeated as soon as the hematological restoration was complete. The treatment was well tolerated and the clinical condition of 31 out of 43 glioblastoma patients improved during the two months after the beginning of the treatment.*

Examination of the results obtained reveals the following characteristics:

1. *A low degree of efficiency of this combination in the treatment of brain metastases, except for breast cancer metastases.*
2. *Absence of complete correlation between the clinical results observed and the cinegammagraphic developments.*
3. *Similarity of the results independent of the initial localization.*
4. *Establishment of a six-months median survival period, with ten patients at present in a state of apparently complete remission, 180–506 days after beginning of the treatment.*

THE CONVENTIONAL treatment applied in malignant brain tumours in adults consists of surgery and irradiation. The prognosis is poor as the mean survival time does not exceed 11 months [1]. The studies of Frankel and German [2] showed that the mean survival time was six months after extensive surgery alone and radiotherapy increases this survival median by about four months.

Experimental results in animals which were later confirmed in man [3–11] have shown the potential value of chemotherapy and led to the development of new therapeutic methods. We disposed of a limited number of effective drugs BCNU, VDR and VM 26.

In the present work, we have analysed the therapeutic results obtained by cyclic and sequential administration of adriamycine, VM 26 and CCNU in 43 patients with non resectable glioblastomas.

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MATERIALS

Forty-three patients were included in this study, beginning in October 1973.

The average age of the 43 patients in this trial (12 women and 31 men) was 52·8 years, the youngest being 20 and the oldest, 71.

Age	*No. patients*
40	5
40–50	9
50–60	17
60–71	12

In 22 patients the tumours were surgically non resectable, either because of their size or their site. Seventeen patients had residual tumours following surgery, and six patients a recurring tumour after a first operation.

TREATMENT

Patients were submitted to intermittent cyclic sequential chemotherapy. Each five-day cycle of treatment included direct intravenous

administration on day 1 of 45 mg/m^2 adriamycine: on days 2 and 3, VM 26 (60 mg/m^2 per day) in 250 ml of isotonic glucose solution, in rapid 1·5 hr infusions, and on days 4 and 5, CCNU *per os*, at a dose of 60 mg/m^2 per day.

Each cycle of treatment was only resumed once hematological restoration was completed, that is, when the leucocyte count was 4/5 of the patient's initial treatment count and the platelet count half the initial count.

Before undergoing the first cycle of chemotherapy, all the patients were given an optimal dose of methylprednisolone which made it possible to reduce the intensity of *intra-cranium* hypertension syndrome manifestations. During this initial phase of treatment, before administration of the first chemotherapy cycle, patients were also given a daily 500 ml perfusion of 100% glucose hypertonic solution, a 500 ml infusion of hypertonic mannitol and 100 mg furosemide, injected i.v.

RESULTS

1. *Tolerance*

All patients underwent the trials considered in this study between two and 17 months ago.

(a) *Hematological disorders.* The intervals between chemotherapy cycles were solely determined on the basis of the time required for complete hematological restoration. *Leucopenia* was moderate and recovery was on the average complete between the 15th and 20th day after the end of the cycle. There were no cases of infection, but *thrombopenia* increased from one cycle to another with increasingly slow restoration. During these trials, *combination chemotherapy* was only resumed once the platelet count had resumed to at least 50% of the pretreatment level. The results, shown in Table 1, indicate a steady increase in the average duration of the interval between chemotherapy cycles. From the first to sixth cycles the duration of this interval increased from 31·6 to 42·2 days, the seventh interval lasting 65·5 days. In all, 170 cycles of treatment were administered to the 43 glioblastoma cases included in the trials and the average interval between cycles lasted 36·6 days.

Thrombopenia was never less than 75,000 mm^3 before the 5th chemotherapy cycle. After the sixth cycle, it dropped to 40,000 in seven patients but there were no cases of hemorrhage. Platelet restoration took a long time in six patients—86·6 days on an average, (ranging from 65 to 125 days) during which clinical relapse occurred.

(b) *Neurological disorders.* The only noticeable neurological complication was intensification of the signs intracranium hypertension during or between treatment cycles. It was observed during the 2nd chemotherapy cycle in 11 cases and during the 3rd cycle in three cases. Such signs disappeared within three days and their intensity was always attenuated by absorption of corticoids. No deaths were imputable to the increase of HT/C.

Table 1. Average increase in the intervals between chemotherapy cycles

Interval No.	No. of patients	Length of interval
1	40	31·6 days
2	33	35·5 days
3	25	36·1 days
4	20	37·8 days
5	16	42·2 days
6	13	38·8 days
7	9	65·5 days
Overall number of treatment cycles for 43 patients*	—	36·6 days

*In six cases, clinical relapse occurred in the course of a very long interval which lasted on an average for 86·6 days.

2. *Evaluation of results*

Intra-cranium tumours develop within a restricted space and their evolution depends on three factors: tumour growth, the development of the peritumoural oedema and the extension to vital neurological centres. Results were evaluated taking into account the interaction of these factors. Each patient included in the trial underwent a general, neurological and biological checkup. The diagnosis was either based on a histological study of the tumour to be operated on or on the correlation between the data provided by the patient's clinical development and by gammaencephalography and arteriography [12, 13].

In 24 cases gammaencephalography was repeated every three months. All the patients were under steroid therapy at the beginning of chemotherapy protocol. Clinical development was judged by the modification of focal neurological signs stabilized from corticoides and the patient's general behaviour, was studied every week, and later at longer intervals.

These signs can be classified into four stages in decreasing order of gravity:

stage IV	the patient is in a coma
stage III	the patient is conscious but his neurological condition requires constant assistance

stage II — the patient is conscious and can look after his basic needs
stage I — the patient can resume all his activities

Improvement was defined as a regression of neurological signs enabling the patient to progress by at least two stages within a minimum of two months.

The final overall assessment of the therapeutical effects was based on the evolution of the survival curve for the entire group of patients included in the trial.

3. *Therapeutic results*

Objective clinical improvement as defined above occurred in 31 patients, that is, in 72% of the cases considered. The clinical condition of 12 others continued to worsen despite the treatment administered (Table 2). Whenever clinical improvement was observed it occurred during the first two months following chemotherapy. Mere stabilization of a patient's initial clinical condition or continued progress after the second month were some of the factors for a bad prognosis that occurred in 12 patients, i.e. in 28% of the cases treated.

Table 2. *Malignant gliomas in adults*

Results observed (Subjective neurological improvements)		
Improvement*	31	72%
Stabilization*	3	7%
Failures	9	21%
Total	43	

*Improvement is defined as a regression of the objective neurological symptoms lasting at least two months.

Twenty-five out of the 31 patients whose condition improved as a result of the treatment were subjected to a regular three-monthly cinegammagraphical checkup (Prof. Ag. D. Ancri). Objective regression of the pathological image was noted in 16 cases; it was complete in five cases and partial in eleven. In all, chemotherapy had an objective effect in 21 out of 25 patients studied but in five cases the image was stabilized. The objective clinical effect noted did not completely correspond to the scintigraphic improvements recorded since scintigraphy showed a growth in the size of the infected site in four patients whose objective clinical condition had apparently improved probably related to a delayed effect of steroid therapy (Table 3).

Table 3. *Malignant gliomas in adults*

Scintigraphic results observed after 3 months' treatment	
Spreading of infected site	4
Stabilization of infected site	5
50% regression	6
50% regression	5
Disappearance of site	5
	25

25 out of 31 cases that improved as a result of the treatment were kept under regular cinegammagraphic observation.

The effect of combined chemotherapy on survival is shown in Fig. 1. The survival median is 180 days. Thereafter, the number of deaths roughly equals the number of patients who survive, taken over the same lapse of time: and at the moment out of 22 patients surviving for more than six months; 12 are in satisfactory condition and 10 are in remission, 180–510 days after treatment.

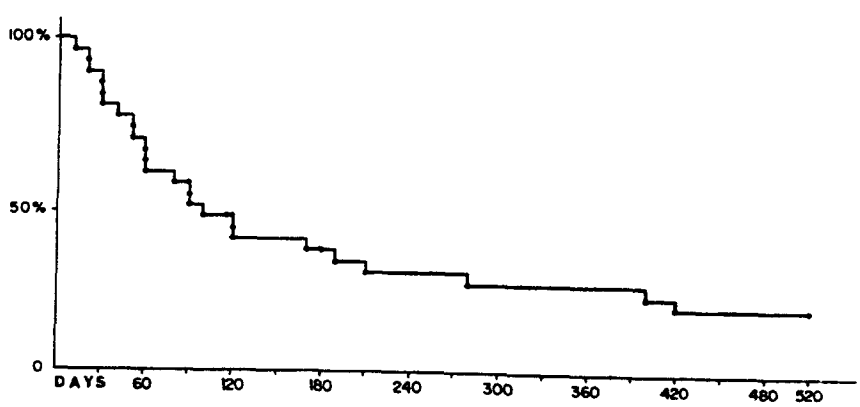

Fig. 1. *Survival curve for* 43 *malignant gliomas patients.*

4. *Factors that may affect chemotherapy results*

The results given in Table 4 show that the initial site of the tumour does not affect the results of chemotherapy, neither does the tumour's extensive but unilateral character have any part in determining its response to drugs. When its initial site is frontal, the survival median is 189 days; when it is temporal, the median is 181 days, when it is parietal, 188 days, and for extensive but unilateral tumours, it is 190 days. It should however be noted that four patients had particularly serious tumours either located in the central grey nucleii or which had spread to the bilateral frontal or callous mass (*corps calleux*). For those patients with right temporal glioblastoma the median survival time is 190 days; and for those left temporal glioblastoma the median survival time is 270 days. This difference led us to suppose that the early diagnosis in relation with the drastic alteration of language, but writing, indicates a smaller tumour, and in this condition a more effective chemotherapy.

Table 4. *Malignant gliomas in adults. Effects of of chemotherapy, classified according to tumour site*

	Frontal	Temporal	Parietal	Extensive tumour
Number of patients	12	14	88	9
Number of survivals on 15.4.1975	5	6	5	3
Survival median	189 days	181 days	188 days	190 days

If one takes into account the treatments received by patients prior to this trial, the survival median for patients having undergone partial tumour *resectioning* is 185 days and may be compared to the 185-day median observed for patients considered inoperable (Table 5).

So far, survival medians have proved identical, but 12 out of 21 patients have survived in the first group and eight out of 22 in the second.

Whatever the results, chemotherapy is regularly applied whenever the patient's hematological state allows. In cases where such treatment continued uninterrupted, *regrowth* occurred mainly after the 6th cycle of treatment (Table 6). In this trial scintigraphic relapses always preceded recurrence of clinical symptoms.

Table 5. *Effects of chemotherapy, according to tumour size*

	Patients included in the trial after partial tumour *exeresis*	Inoperable tumour patients included in the trial
Number of patients	71	22
Survival median on 15.4.75	185 days (30 to 400 d)	188 days (40 to 506 d)
Number of survivals	12	8
Average survival periods for diseased patients	154 days	168 days

Table 6. *Malignant gliomas in adults. Effects of chemotherapy according to tumour site*

	Tumour site	
	Right temporal	Left temporal
Survival median	190 days (40 to 400 d)	270 days (134 to 446 d)
Number of patients	8	6
Number of survivals	3	3

DISCUSSION

Forty-three patients with glioblastoma and 30 patients with multiple brain metastasis were treated by sequential administration of adriamycine, VM 26 and CCNU. For the patients suffering from glioblastoma, the clinical response rate was 72% and the objective cinegammagraphic response, 58%.

Taking into account the criteria applied to selection of patients for this non comparative trial, the results are encouraging as far as the duration of survival is concerned. For a series of comparable patients treated by simple outside decompression, the survival median was two months [2, 14]. In our series, 90% of the patients survived into the 2nd month, the overall survival median being six months with the extreme limit up to the 17th month. In the case of four patients who have now had over a year's treatment, there have so far been no neurological or cinegammagraphic consequences due to their initial tumour.

High rates of objective response (50%) were obtained with the administration of BCNU alone [15, 8, 10]. CCNU has a comparable efficiency but seems easier to handle and less

toxic [16, 17]. The results obtained by Sklansky using VM 26 [6] indicate that the drug is especially effective in cases of resistance to nitrousereas. Earlier trials using combinations of individually active drugs (VCR and BCNU) proved disappointing in the treatment of glioblastoma.

A comparison of the results obtained in this trial as regards survival, based on reference to groups treated respectively by surgery only and by surgery combined with radiotherapy shows that for those of our patients who did not respond to other forms of treatment, such combinations of drugs made it possible to achieve results similar to those obtained from a group for which the prognosis was apparently less grim. Using this combination, D. Fewer[18] obtained less satisfactory results than those obtained by the use of BCNU alone.* Using a combination of CCNU, vincristine and methotrexate, J. Hildebrand [19] reported results comparable to those achieved by administering CCNU alone. A non-comparative trial combining VM 26 and CCNU [20] led to a neurological improvement in 60% of patients and a final six months survival median [21]. The small number of patients involved in this trial did not enable its results to be compared to those obtained by administration of CCNU alone.

However, our subsequent trial included 43 glioblastoma cases, and evaluation of the results bring out the following five points for discussion:

(1) Neurological improvement always occurred during the first month of treatment and the absence of effect after the second cycle is the cause of a bad prognosis. Any rises in intracranium hypertension due to chemotherapy were easily kept under control by the treatment and did not cause any deaths.

(2) There was no absolute correlation between the clinical effects and objective cinegammagraphic regression observed. Twenty per cent of the patients who had improved neurologically showed a deterioration on scanning. This discrepancy between results had earlier been noted by Fewer. Apart from cases where chemotherapy did not modify the tumour's clinical development, all patients in whom showed an increase in or only stabilization of the initial tumour size died within the first six months. Consequently, there seems to be an exact correlation between prognosis during chemotherapy and the modifications observed in cinegammagraphy [16, 18].

(3) According to the results given in Tables 5 and 6 initial tumour size does not seem to alter the treatment's effect on survival. The different tumour sites appear to be sensitive to chemotherapy to an identical degree. A significant difference was, however, noted between the two groups of patients whose tumours originated in the left and right temporal zones respectively.

(4) Although it is still too early to formulate a final opinion the survival median observed for the 43 glioblastoma patients who were given the triple drug combination specified above and were all initially inoperable may be compared to the median reported for earlier series of patients treated by radiotherapy after partial or no resectioning [2].

*J. Hildebrand [19], using a combination of CCNU, vincristine and MTO, reported results comparable to those achieved by administrating CCNU alone.

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Chemotherapy of Bronchogenic Carcinomas by a Combination of Cyclophosphamide, Methotrexate, Vincristin and Bleomycin

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Abstract—*Thirty-seven patients with primary unresectable bronchogenic carcinoma were treated by a combination, ("E.M.O.B."), of cyclophosphamide (40 mg/k on day No. 1, or 13·3 mg/k × 3 consecutive days), methotrexate (7·5 mg/m2/d × 6 d, given orally for 6 consecutive days from day 21 to 26), Folinic Acid (15 mg/m2, IM, on day 27 at 8 p.m.). vincristin (0·7 mg/m2, IV at 8 a.m. on days 28 and 31) and bleomycin (10 mg/m2, IM at 2 p.m. on days 28 and 31, i.e. 6 hr after vincristin. The response was evaluated on day 48 and a new course started on day 49 if a regression or a stabilisation had been obtained.*

The 37 cases include 9 small anaplastic carcinomas, 24 squamous cell and large cell anaplastic carcinomas, and 4 cases of undetermined type. Thirty-one patients received almost 1 course of the schedule. Among them, 1 had a complete regression, 5 a regression of more than 50%, 6 a regression of less than 50%, 14 a stabilisation. There were 11 failures. Among 12 objective responders (39%) there were 4 squamous cell carcinomas (22%, median duration: 5 months), and 8 oat cell carcinomas (89%, median duration: 4 months). Four out of 37 patients (10%) were alive after 1 yr.

The toxicity was evaluable in 32 cases representing 77 courses of the schedule: in 3 cases a severe toxicity led to an interruption of the treatment; strong (4 cases) or tolerable (15 cases) toxicity included nausea, vomiting and marrow depression. In 10 cases there were no toxic phenomena (31%).

The over all results of this combination are not significantly better than those that may be obtained with cyclophosphamide or methotrexate as single agents or with vincristin and bleomycin in combination.

INTRODUCTION

AT THE present time, the results obtained with chemotherapy in advanced bronchogenic carcinomas, are poor by both the rate of regression and the duration of response and survival gain. We attempted to associate 4 drugs the efficacy of which is well known though limited: cyclophosphamide [1], methotrexate [2], and vincristin followed 6 hr later by bleomycin [3, 4].

MATERIAL AND METHODS

1. *Treatment schedule*

All the patients received the following treatment:

—Cyclophosphamide, 40 mg/kg, by i.v. perfusion on day no. 1 (or 13·3 mg/kg/day, i.v. on days No. 1, 2 and 3),.

—Methotrexate, *per os*, 7·5 mg/m^2/day × 6 consecutive days, from day No. 21 through day No. 26,

—Folinic Acid, 15 mg/m², was administered intramuscularly at 8 p.m. on day No. 27,
—Vincristin, 0·7 mg/m², i.v., on day No. 28 at 8 p.m.,
—Bleomycin, 10 mg/m², i.m., on day No. 28 at 2 p.m.,
—Vincristin, and bleomycin 6 hr later, were repeated at the same dosage on day No. 31.

This association was called E.M.O.B.*

The response was evaluated 15 days later on day No. 48. If there was no progression of the disease, or a tumor regression, a second course was started on day No. 48; on the contrary, the treatment was stopped if the disease had progressed. Blood counts were performed on days No. 1, 21, 28 and 47: in a few cases, drug doses were reduced to 50% if there existed a moderate hematological toxicity but there was no protocol rule on that topic.

2. *Patients*

From May 1, 1973, through December 31, 1974, 42 consecutive patients entered in that study. All had a primitive bronchogenic carcinoma in an evolutive and advanced state. Their ages ranged between 42 and 81 (mean: 59; median: 62). Three of these patients were women.

Eighteen had been previously treated by either surgery (6 patients), radiotherapy (4 patients), surgery and radiotherapy (3 patients) or surgery, radiotherapy and chemotherapy (5 patients); 24 had not been previously treated.

In five cases, the first course of chemotherapy was arbitrarily suspended. Owing either to a too poor general condition in 3 cases, a too far advanced age (81 years) in one case or to the patient's own refusal in one case. These five cases were excluded.

The 37 remaining patients were included, even if the first course was not completed.

The histologic type is presented in the first table.

Table 1.

Histologic type	Number of patients
Oat cell carcinoma	9
Epidermoid carcinoma and large cell anaplastic carcinoma	24
Undetermined	4
Total	37

*"E. M. O. B." from ENDOXAN (cyclophosphamide), methotrexate, ONCOVIN, bleomycin.

Among these 37 patients, six died during the first course and were nevertheless included in the study of results. Three patients were living at the end of the study (3, 4 and 18 months after the beginning of the first course). One patient was lost to follow up after 7 months of treatment.

RESULTS

1. *Evaluation criteria*

Survival and toxicity data were estimated from the 37 treated patients. The response rate was evaluated from 31 patients in which almost one course was completed and who therefore were defined as "adequately treated patients".

Response criteria were as follows:
—regression of more than 50%: when the products of two perpendicular diameters of the tumor as measured on frontal and sagittal X-ray films, was reduced by more than 50%;
—partial regression of less than 50%: when the radiologic improvement was obvious but not accurately measurable;
—no progression: this was applied to the cases with an atelectasis in which we obtained simultaneously a radiologic and stable functional improvement. The other cases were considered as progression. Nevertheless, when there was a superficial adenopathy the result was evaluated from its volume variations.

2. *Response data* (*Table* 2)

Among 31 treated patients, we obtained 6 regressions of more than 50%; 6 partial regressions of less than 50%. Thus, the objective response rate was 12/31 (39%). But we obtained only 4 objective regressions out of 18 epidermoid and large cell anaplastic carcinomas (22%), when we obtained 8 objective responses in 9 adequately treated patients with oat cell carcinomas (89%).

The median duration of objective responses in epidermoid carcinomas was 5 months. The median duration of the response including regressions and stabilisations was 3·7 months.

The best responses were obtained in small cell anaplastic carcinomas (1 complete regression, 7 partial regressions and 1 stabilisation). The median duration of objective responses was 4 months.

3. *Survival*

Out of 37 patients, only 4 (10%) have survived one year or more. The median survival was 5 months for the whole; 7·7 months for the responders; 5·9 months when the re-

Table 2.

Histologic type	Total (1)	n	Complete regression	Regression > 50% (+)	< 50% regression	Objective regression rate	Stabilization	Progressive disease
Epidermoid and large cell anaplastic carcinoma	24	18		2 (11%) (8%)	2 (11%) (8%)	4 (22%) (17%)	10 (55%) (42%)	4 (22%) (17%)
Oat cell carcinoma	9	9	1 (11%)	3 (33%)	4 (44%)	8 (89%)	1 (11%)	
Undetermined	4	4					3 (75%)	1 (35%)

(1) Number of treated patients.
n Number of adequately treated patients.
(+) The 1st number in the brackets applies to the adequately treated patients; the 2nd number applies to the total number of patients.

sponse was "no progression"; and 1·3 months for the non responding patients.

Table 3.

Response type	Number of patients	Median duration of survival (months)
All patients	37	5
Objective > and < 50%	12	7·7
No progression	14	5·9
Progressive disease	11	1·3

4. *Toxicity*

Toxicity data were available in 32 out of 37 treated patients; these data were missing in five patient files. The toxic events were classified in three groups:

(1) acceptable toxicity.
(2) strong toxicity.
(3) accidents.

The criteria of toxicity are listed in the legend of Table 4. A strong toxicity was observed in 4 patients and severe accidents occurred in 3 patients. Though important this toxicity may appear, in fact, out of 77 courses of the EMOB protocol, we observed no toxicity at all in 40 courses (52%). The three severe accidents are to be included in the same total number of courses, i.e. 3/77 (4%). The toxicity was the same in the different histologic types.

The observation of the 31 patients who had completed at least one course was used to study the toxicity of the components of the association (E.M.O.B.). The methotrexate was responsible for the most frequent and the most severe manifestations (digestive tract troubles, stomatitis).

Table 4.

	No toxicity (1)	Acceptable toxicity (2)	Strong toxicity (3)	Toxic accidents (4)
Numbers of patients	10	15	4	3
Percentage	31%	47%	13%	9%

(1) No physical or biologic sign of toxicity. Yet a transitory leucopenia, almost constant after the cyclophosphamide injection, was not considered as a toxicity sign when it had spontaneously regressed eighteen days later.
(2) Moderate and reversible physical or hematological symptoms.
(3) Severe discomfort (vomiting, stomatitis) and/or deep or prolonged cytopenia. Treatment might be delayed but not definitely stopped.
(4) Treatment had to be definitely stopped and/or was partly responsible for the patient's death.

DISCUSSION

The rationale for the EMOB schedule was the following: the initial loading dose of cyclophosphamide was employed as to reduce tumor volume and, as a consequence, to recruit cells from Go to the cell cycle. Then methotrexate was assumed to be more active. After the six day course of methotrexate, Folinic Acid was administered with the aim to "unblock" cells that could be blocked in the S phase, and to synchronize them. We arbitrarily assumed that 12 hr later these synchronized cells had reached the G2-M period which is the optimal phase for the action of vincristin then bleomycin [3, 4].

In that schedule, the toxicity of each drug was not cumulated for there was a sufficient interval between cyclophosphamide and methotrexate.

Our actual results are not superior to those that may be obtained with cyclophosphamide alone [1] or high dose methotrexate alone [2]. They are not very different from those that were obtained with vincristin and bleomycin in combination [4]. They are inferior to the results obtained with a combination of vincristin, CCNU and Fluoro-Uracil by Pouillart *et al.* [5].

The response rate of EMOB schedule in oat cell carcinomas is likely to be obtained with a number of drug combinations.

We are now studying a combination of the same drugs (cyclophosphamide, methotrexate, Folinic Acid, vincristin, bleomycin) with quite a different schedule; the drugs are administered in a 48 hr period, taking in account the potential cumulated toxicity. This new schedule is randomly compared to the association of vincristin, CCNU and 5 FU.

The study is in progress and no preliminary data are available at the present time.

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Synchronization Recruitment Chemotherapy in Non-Hodgkin Hematosarcomas

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Abstract—*Thirty-three patients with poorly differentiated, diffused lymphosarcoma or reticulosarcoma, stages III and IV were treated by a potentiating chemotherapy. The cycles comprised 25 mg/m² Adriamycin on day 1, 40 mg/m² VM 26 on day 2, 300 mg/m² cyclophosphamide and 40 mg/m² prednisone from day 3 to day 6. Tolerance was excellent in most cases. We note 61 % complete and incomplete remissions, 27 % regressions less than 50 % and 12 % failures.*

INTRODUCTION

CHEMOTHERAPY represents the first induction treatment of dissiminated lymphosarcomas and reticulosarcomas. We use it more and more often since modern methods of clinical, biological, radiological, scintigraphical and surgical investigations have shown that many types, classified in the past as local or regional, were in fact disseminated, invading lymph nodes above and below the diaphragm and/or different viscera.

Several substances are effective in these diseases. We have used sequentially adriamycin, epipodophyllotoxin, cyclophosphamide and prednisone in the aim of cell recruitment and synchronisation [1, 2] according to the protocol proposed by G. Mathé at Villejuif. The dosages of adriamycin and prodophyllotoxin (VM 26) had to be modified due to the high mean age of our patients.

1. PATIENTS AND METHODS

1. *Patients*

Thirty-three patients (17 men and 16 women) whose ages ranged from 26 to 86 (median 66) were treated by this chemotherapy. All the patients were in the perceptible phase of their illness: 19 in the 1st phase, 12 in the 2nd, 1 in the 3rd and 1 in the 4th. Histologically and topographically (Table 1) we have noted 18 diffused, poorly differentiated lymphosarcomas, 9 stage III and 9 stage IV; and 15 diffused, poorly differentiated reticulosarcomas, 3 stage III and 12 stage IV. Apart from the usual clinical examination, pre-therapeutic investigation of these patients included: chest X-ray, with mediastinal tomography, abdominal lymphography, spleen, liver and bone scintigraphy, skeletal survey, barium meal, bone-marrow aspiration and biopsy, liver biopsy, immunoelectrophoresis, blood cell count, erythrocyte sedimentation rate and immune exploration. Laparotomy was not used due to the old age of most patients.

Table 1. *Chemotherapy of lymphosarcoma and reticulosarcoma*
Classification of patients

Diffuse poorly differentiated			
Lymphosarcoma stage:		Reticulosarcoma stage:	
III	IV	III	IV
9	9	3	12

2. *Chemotherapy*

As indicated in Table 2, the treatment comprises 6-day-cycles with on day 1, 25 mg/m² adriamycin i.v., on day 2, 40 mg/m² VM 26 i.v. infusion and from day 3 to 6, 300 mg/m² cyclophosphamide i.v. and 40 mg/m² prednisone orally. This treatment is taken again after hematological restoration if the leucocyte level is above 4000/mm³ and platelet level above 100,000/mm³. Hematological restoration usually occurs between 15 and 24

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days and the interval between cycles is about 21 days. The number of cycles varies from 1 to 10 with a mean of 4.

Table 2. Chemotherapy of lymphosarcoma and reticulosarcoma

Day 1	Adriamycin	25 mg/m²	i.v.
Day 2	VM 26	40 mg/m²	Perf.
Day 3–6	Cyclophosphamide	300 mg/m²	i.m. or i.v.
	+ Prednisone	40 mg/m²	

Interval 21 days.

2. RESULTS

2.1. *Toxicity*

The chief toxic effects are mentioned in Table 3. Leucocytes were constant and it was only in 9 cases that the rate was under 2000/mm³. Thrombocytes were also constant and only 3 cases were under 50,000/mm³. Other toxic effects are essentially asthenia, nausea, alopecia. In one patient, after 2 cycles of chemotherapy, we observed symptoms evoking viral encephalitis which caused death. Unfortunately necropsy was not possible. No cardiac toxicity was observed, but adriamycin dosage was low (25 mg/m²) in view of the patient's age. In 8 cases/33, no clinical or hematological toxicity was observed. Overall tolerance was excellent and we had no difficulty in applying a ten-cycle programme of treatment even in patients whose general condition on arrival was very poor.

Table 3. Chemotherapy of lymphosarcoma and reticulosarcoma

Toxicity in 33 patients	
Leucopenia < 2000/m³	9
Thrombopenia < 50 000/m³	3
Asthenia	10
Nausea	8
Alopecia	4
Lethal encephalitis	1
Cardiac toxicity	0
No toxicity	8

2.2. *Antitumoral effectiveness*

Results are shown in Table 4. We have seen 4 complete remissions, with complete disappearance of clinical, radiological and biological evidence. We have obtained 18 incomplete remissions of more than 50% with a reduction of nearly all lesions. In 9 cases, regression was less than 50% and in 4 cases, the treatment was a failure. In fact, we have observed 61% complete and incomplete remissions, 27% regressions less than 50% and 12% failures.

Results are better in lymphosarcomas than in reticulosarcomas. In the first type, we have obtained 67% complete and incomplete remissions, 33% regressions less than 50% and no failure; in the second type, 53% complete and incomplete remissions, 20% regressions less than 50% and 27% failures.

In these two types of hematosarcomas, good results have been observed not only in disseminated, superficial and deep lymph nodes (mediastinal and lomboaortic) but also in stage IV with visceral lesions (52% complete and incomplete remissions) and in 9 cases, more or less complete regressions of liver involvement was observed; in 2 cases bone lesions dis-

Table 4. Chemotherapy of diffuse poorly differentiated lymphosarcoma and reticulosarcoma stages III and IV. Results

		Complete remission	Remission > 50%	Regression < 50%	Failure	Total
Lymphosarcoma	III	1	5	3	0	9
	IV	—	6	3	0	9
	Total	1	11	6	0	18
Reticulosarcoma	III	2	1	—	—	3
	IV	1	4	3	4	12
	Total	3	5	3	4	15
Total LS+RS		4	16	9	4	33

appeared, in 3 cases pleural or pulmonary lesions and in one case sub-cutaneous disseminated nodules.

The maximum therapeutic effect was observed at the 1st cycle in 2 cases, at the 2nd cycle in 4 cases, at the 3rd cycle in 12 cases and at the 4th cycle in 2 cases.

No statistical evaluation can yet be made regarding the duration of incomplete remissions because patients have undergone subsequent radiotherapy to complete the remission. As for the 4 complete remissions, in one case of lymphosarcoma, stage III the patient was lost to follow-up after 6 months; in 1 case of reticulosarcoma, stage IV, we observed a complete remission without treatment lasting 24 months and in the 2 other patients with reticulosarcoma, stage III, remission was still in progress after 6 and 12 months.

3. DISCUSSION

Many synchronisation chemotherapies are currently in use with variable results, and often toxic effects, particularly hematological, which limit the length of the treatment and make the problem of further treatment more difficult.

The therapeutic protocol studied in this work seems to us of great interest because of its excellent tolerance which has allowed us to administer up to 10 cycles in patients over 60, after sometimes prior administration of other chemotherapy or application of radiotherapy. Antitumoral effect is remarkable since it was observed on superficial and deep adenopathies, but also on visceral and especially hepatic lesions. In lymphosarcomas, no failure was observed on the contrary of reticulosarcomas often more resistant to anti-cancerous treatments. Our results were somewhat inferior to those of Misset *et al.* [3] but our dosages of adriamycin and VM 26 were lower due to our patients' age. Some years are still necessary to tell us if we can improve the prognosis of these diseases which modern means of investigation show are frequently disseminated.

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Pharmacokinetics of Adriamycin and Adriamycin–DNA Complex in L1210 Mice and Men

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Abstract—*Adriamycin when linked to desoxyribonucleic acid gives higher plasma concentrations than adriamycin injected at the same dose.*

In mice, under certain conditions, the therapeutic activity of ADM–DNA is higher than ADM.

In men, both forms of the drug induce the same toxicity but for a C × t bigger for ADM–DNA than for ADM.

INTRODUCTION

ADRIAMYCIN is an anthracycline antibiotic which induces objective responses in many solid tumors and hematologic malignancies [1–3]. Its mechanism of action seems to be the binding of the drug to cellular DNA by intercalation between base pairs, and inhibition of RNA synthesis [2, 3].

The therapeutic usefulness of adriamycin is however restricted by its toxicity on the bone marrow and heart muscle. Trouet *et al.* [4, 5], using the concept of lysosomotropic cancer chemotherapy, have been able to decrease toxicity and to increase activity in L1210 leukemia in DBA_2 mice by binding adriamycin and DNA. The concept of lysosomotropic chemotherapy implies that an antitumoral drug firmly linked to an endocytisable and digestible carrier will penetrate only cells with a high endocytic activity. In the cell, the carrier will be digested by the lysosome and the drug will become free to diffuse. Cells which do not possess both high endocytic and mitotic activities would then be protected from the action of the drug.

The present investigation was performed to compare the pharmacokinetic properties of adriamycin when infused alone or complexed with DNA.

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MATERIAL AND METHODS

The adriamycin–DNA complex (ADM–DNA) was prepared as described by Trouet *et al.* [4] by mixing adriamycin and a DNA solution (Herring sperm type VII; Sigma, Saint Louis) with a Molar ratio of DNA mononucleotides to adriamycin equal to 20. The apparent binding constant of ADM to DNA is $0{\cdot}41 \cdot 10^6\ M^{-1}$ [5].

L1210 leukemia was obtained from Trouet [5] and maintained in DBA_2 female mice (Ch. Rivers Laboratory). Ascites tumor cells were counted in a hemocytometer and diluted in Hank's balanced solution. 0·1 ml (10^5 cells) was injected i.v. into DBA_2 mice, 8–10 weeks of age, weighing 20–24 g.

A single dose of adriamycin (ADM) or ADM–DNA (6 mg/kg of adriamycin in both cases) was injected intravenously 24 hr after L1210 cells inoculation. At selected intervals, venous blood samples were collected from the inferior vena cava of anesthetized animals. Heparinized pooled blood of 3 groups of 6 mice was centrifuged immediately and the plasma was frozen at −20°C for a maximum of one month. In men, ADM or ADM–DNA was administered by intravenous infusion to 2 groups of 4 patients at the dose of 75 mg/m^2 of body surface area in 1–3 hr. Ten milliliter samples of heparinized venous blood were collected before drug administration and at specified intervals thereafter. The samples were immediately centrifuged, and the plasma

separated, frozen and kept at −20°C for a maximum of one month.

Total plasma fluorescence assay

Total plasma fluorescence was assayed by a modification of the method of Bachur *et al.* [6]. Frozen plasma was thawed and sonicated for dispersion of insoluble material. Absolute ethanol (1·5 ml) and hydrochloric acid 2 N (0·45 ml) were mixed with each plasma sample (1 ml).

The mixture was allowed to stand at 4°C for 24 hr. The samples were centrifuged at 50,000 × *g* in a Beckman Spinco L265B ultracentrifuge, for 25 min and the fluorescence of the plasma acid alcohol extracts was determined in a Zeiss PMQ3 spectrophotofluorimeter.

An activation wavelength of 465 mμ and an emission wavelength of 595 mμ were used. The concentration of adriamycin equivalents in the plasma samples were determined by direct comparison of the relative fluorescence intensity of the plasma extracts with a calibration curve.

A standard curve was performed for each set of estimations. A corresponding blank containing no drug was also carried through the above procedure, and the relative fluorescence intensities were corrected by subtracting the value obtained for the blank (endogenous fluorescing materials) from that of each of the extracts. All samples were analyzed in triplicate. All results are given in adriamycin equivalents.

RESULTS

1. *Recovery studies*

Standard curves obtained when the ADM and ADM–DNA were added to plasma (0·01–5 μg/ml) were identical with curves obtained by adding adriamycin directly to plasma supernatant obtained after protein precipitation. Acid–alcohol extraction of standard solutions of ADM and ADM–DNA in plasma resulted in a 90–100% recovery of both drugs above 0·01 μg/ml concentration in plasma. The relationship between the relative fluorescence intensity and the concentration of ADM and ADM–DNA in the standard solutions is linear in the range of experimental data.

2. *Pharmacokinetics in mice with L1210 leukemia*

Plasma concentrations of ADM equivalent are displayed in Fig. 1. There is a large difference between plasma concentration during the first two hours after drug injection. However, after the third hour, both drugs give the same plasma concentrations.

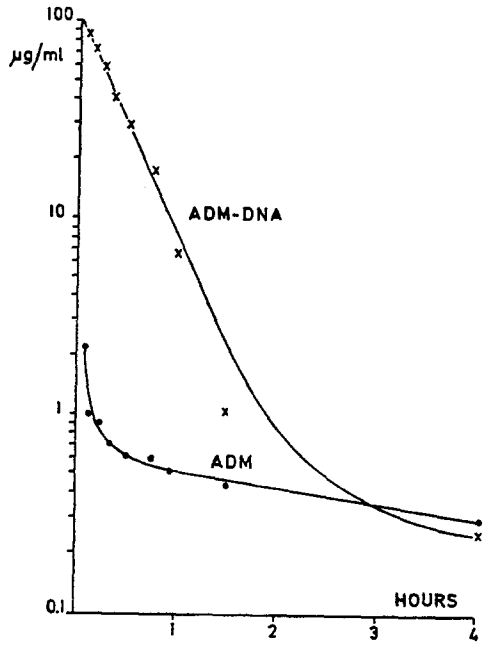

Fig. 1. Plasma concentrations of ADM equivalents in DBA/2 mice with L1210 leukemia after i.v. injection of 6 mg/kg ADM or ADM–DNA. Least square curves and means of experimental values.

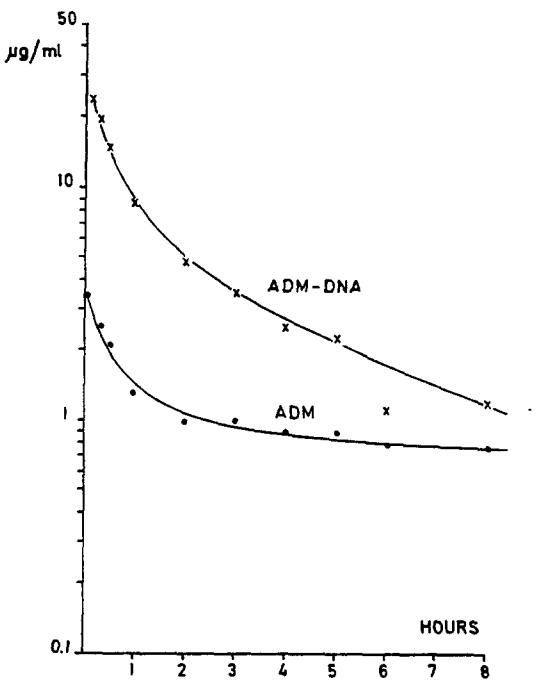

Fig. 2. Plasma concentrations of ADM equivalents in men after i.v. infusion of 70 mg/kg. Least square curve and means of experimental values.

3. *Pharmacokinetics in men with various neoplasia*

Plasma levels of ADM equivalents after cessation of the infusion are shown in Fig. 2. ADM–DNA in spite of a longer infusion time,

gives plasma concentrations of ADM equivalents about 8 times higher than ADM just after cessation of the infusion. At the 10th hr, the same plasma concentration of ADM equivalent is seen with both forms of the drug.

4. *Toxicity and therapeutic efficacy*

It has been shown that ADM–DNA is less toxic than ADM in mice, and an increased activity in some sublines of leukemia L1210 has been demonstrated [7]. In solid tumors, the complex, when given at the dose of 6 mg/day for 5 days, was more active than adriamycin alone in Melanoma B16 when administered i.p. In Lewis Lung carcinoma and L1210 transplanted s.c., adriamycin was shown to be superior to the complex [8, 9].

In men, a phase I study suggested that the toxicity induced by the complex was similar to that observed with the free drug [10].

DISCUSSION

In mice and men, plasma levels of ADM and its metabolites are strikingly different for both forms of the drug. The most important factor playing a role in these differences seems to be the size of the macromolecule of ADM–DNA which hampers the passage through the capillary barrier. After a few hours, plasma levels of ADM equivalents are the same for both drugs in mice and men, as if the complex ADM–DNA is dissociated either by pinocytosis or by the action of circulating desoxyribonuclease.

Therapeutic results observed in mice [8, 9] have indicated that ADM–DNA is superior to ADM in intraperitoneally transplanted malignancies whereas the contrary is seen in solid tumors, although the optimal schedule was not used for ADM. This can be interpreted as due to the slow diffusion of ADM–DNA from well-perfused compartment to the tissues and the tumors. This hypothesis has been confirmed in the case of the isolated perfused heart of the rat where ADM–DNA was shown to be less toxic than ADM [11].

In men, no controlled therapeutic trial has been conducted so far. A recent phase I study [10] suggested that hematologic toxicity is identical with both drugs in spite of a higher $C \times t$ for ADM–DNA.

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Intralymphatic Immunization: Current Status*†

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Abstract—*A progress report and the research plan for a new method of antitumor immunization using the intralymphatic route are presented. A total of* 205 *intralymphatic infusions of intact cellular vaccines have been performed in normal and tumor-bearing dogs.* In vitro *and* in vivo *experiments on normal dogs demonstrated that intralymphatic immunization produces a more rapid and more intense cytotoxic cellular immune response compared to the subcutaneous route. Experiments in tumor-bearing dogs showed reduction or stabilization of tumor mass after intralymphatic infusion of irradiated malignant cells. These data indicate that intralymphatic immunization may be the method of choice for stimulating strong cellular immune responses to weakly immunogenic materials.*

INTRODUCTION

THE FIRST attempt of antineoplastic intralymphatic immunostimulation by infusion of presumedly immunogenic material (irradiated autochthonous tumor cells) was performed on September 8, 1972 on a 75-yr-old patient with disseminated melanoma. Three consecutive infusions via the same afferent lymphatic vessel were done 3 and 4 days apart. During subsequent weeks, all metastases continued to grow with the exception of a nodule of the right thigh which was in the infused area. The patient expired 3 weeks later.

This attempt in a human patient, which was made under exceptional circumstances, stimulated further investigation of the intralymphatic route of immunostimulation as a potentially effective mode of antitumor immunotherapy. However, since neither the benefits nor hazards of this method were known, it was not appropriate to proceed further in humans without undertaking relevant preclinical experiments on animals. Such studies have been in progress since January of 1974 in the Division of Radiation Therapy at UCLA [1, 2, 3].

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†Presented at the First Congress of the Medical Oncology Society at Nice on December 3, 1975 by Professor C. M. Lalanne, Director, Centre Antoine Lacassagne, Nice, France.

MATERIAL AND METHODS

All experiments have been done on randomly-bred normal and tumor-bearing dogs which were selected for the following reasons:

1. Dogs develop *spontaneous* malignant tumors that are similar to human malignancies.
2. Injection via the intralymphatic route is technically almost as easy as it is in humans.

Standard lymphangiography techniques were used for cannulation and infusion of the extremities.

RESULTS

1. *Experiments on normal dogs*

Differential immune responses to immunogenic material injected intralymphatically, subcutaneously, or intradermally have been studied on 22 normal, weight-matched dogs.

Evaluation of immune responses to a malignant cell vaccine. Direct cell-mediated cytotoxicity, complement-dependent antibody cytotoxicity, and skin test reactivity to a xenogeneic leukemia cell line was serially assessed in 16 dogs divided into 4 groups which had received respectively:

—Intralymphatically, a suspension of 10^8 leukemia cells.
—Intralymphatically, medium only.
—Subcutaneously, 10^8 leukemia cells.
—Subcutaneously, medium only.

Results of both primary and secondary immunizations showed that cell-mediated

immune responses were preferentially accelerated and significantly greater in the groups which had received leukemic cells intralymphatically.

Evaluation of responses to BCG. In this study, 2 groups of 4 dogs received respectively BCG (Glaxo) intradermally and intralymphatically. The responses were evaluated clinically by tuberculin skin testing, and the sera were serially sampled to assess antibody against BCG.

All dogs which received the BCG intralymphatically developed large regional lymphadenopathies which persisted for several months Two dogs in the "intralymphatic" group developed positive skin tests at 11 and 23 days, and skin test became positive at 45 days in the "intradermal" group. The level of antibodies is not known yet; all the sera will be assessed at the same tine.

2. *Experiments on tumor-bearing dogs*

A. Randomized study of Stage III malignant lymphomas was undertaken in which one group (7 cases) received intralymphatic infusions of irradiated autochthonous tumor cells. The second group (4 cases) received intralymphatically the medium only.

The lymphadenopathies have been found to routinely decrease in size at least temporarily in Group 1 but continue to grow progressively in Group 2. Thus far, the mean survival is 42 ± 30 days in Group 1 and 13 ± 6 in Group 2.

B. Nonrandomized study of intralymphatic immunization in solid tumors:

1. Irradiated autochthonous, allogeneic, or xenogeneic tumor cells were repeatedly infused via the intralymphatic route in:

Two cases of pulmonary metastasis from breast adenocarcinoma;

Two cases of pulmonary metastasis from soft tissue sarcoma;

One case of epidermoid carcinoma of the upper respiratory tract.

All cases showed reduction or stabilization of tumor mass.

2. BCG was administered intralymphatically in 2 cases of melanoma. Both cases expired within 10 days of the first infusion with fulminant tumor growth.

DISCUSSION

From these studies involving 205 intralymphatic infusions in 41 dogs, the following points can be made:

1. The intralymphatic route of administration not only is technically applicable but can be used repetitively if necessary. Up to 9 infusions at the same site have been performed over a 5-month period in the same animal.
2. No side effects, including anaphylactic or allergic reactions, have been observed following intralymphatic injection of autochthonous, allogeneic, or xenogeneic tumor cells.
3. Cellular immune responses are quantitatively greater following intralymphatic immunization than the ones elicited by the subcutaneous immunization.
4. A reduction or stabilization of tumor size has always been observed after intralymphatic infusion of irradiated tumor cells.
5. These tumor reductive responses are not due to a direct toxic effect since they were observed in remote areas (pulmonary metastasis, carcinoma of the upper respiratory tract, distant lymphadenopathies).

CONCLUSION

Our findings indicate that intralymphatic injections may be the route of choice for the immunotherapeutic administration of *weakly immunogenic* cellular materials and that no adverse side effects or complications have thus far been associated with this method of immunostimulation.

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Are Circulating Null Cells in Patients Submitted to Long-Term Immunotherapy, Related to K Cells?

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Abstract—*In an attempt to elucidate a possible mode of action of immunotherapy in acute lymphocytic leukemia, we studied the mononuclear subpopulation in a group of patients with ALL in remission on long-term immunotherapy, a similar group on chemotherapy and a group of normal individuals.*

Comparison of the three groups demonstrated a significant increase in the number and percentage of "null cell" in the immunotherapy group relative to the two others. Although the nature of these cells is unknown, the possibilities that they represent persistent abnormal elements, circulating stem cells, "K" cells, or modified T, B or monocytic cells are raised.

INTRODUCTION

IN THE last decade, many reports have attested to the clinical benefits of immunotherapy in the treatment of malignant diseases. Extensive reviews of these publications have recently appeared [1, 2]. Although experimental evidence regarding the mechanism of action of BCG and/or inactivated tumour cells on animal immune systems is abundant [3–7] consistent data in humans is still scanty.

The purpose of the present study is to evaluate the effect of such a therapeutic programme on the circulating mononuclear cell population in patients in complete remission from acute lymphocytic leukemia (ALL). To this end, we compared a group of patients on long term immunotherapy to a similar group receiving standard maintenance chemotherapy and to a group of normal individuals.

MATERIAL AND METHODS

Patients

Thirty-one patients with ALL currently in remission were studied. Twenty of these were receiving immunotherapy and 11, chemotherapy at the time they were tested. Immunotherapy consisted of fresh living BCG (Pasteur) administered by scarification at intervals ranging from once weekly to once monthly and irradiated, formalized allogenic blast cells (10^8) administered by scarification monthly [8]. Chemotherapy consisted of weekly methotrexate (IM 15 mg/m^2) and cyclophosphamide (200 mg/m^2) plus daily 6-Mercaptopurine (P.O. 50 mg/m^2). Precise dosage was adjusted weekly according to the level of neutrophils. These patients were compared with 9 normal subjects as controls.

A summary of the age and sex distribution, plus the duration of maintenance treatment is presented in Table 1. Although the patients investigated were drawn from several different protocols operational over the years, all such protocols consist of a period of post induction chemotherapy followed by immunotherapy. Accordingly the immunotherapy group had received a variable period of post-remission chemotherapy and then a period of immunotherapy ranging, as seen on Table 1, from 13–96 months. The patients in the chemotherapy group are those who have not yet completed the chemotherapy phase of the current ALL protocol and have been treated for periods ranging from 3 to 14 months.

Laboratory procedures

Total and differential white cell counts were performed on all subjects. The absolute number of each category of cells was calculated by multiplying the percentages obtained in the following procedures by the absolute number of mononuclear cells.

Table 1. Study groups (A.L.L. in complete remission)

	Number of patients	Sex	Age (mean + range)	Duration of maintenance treatment (mean + range)
Chemotherapy	11	10 M 1 F	10·1 years (5–31)	8·9 months (3–14)
Immunotherapy†	20	7 M 13 F	11·5 years (4–21)	28·2 months (13–96)
Normals	9	4 M 5 F	30 years (20–50)	—

†Post-chemotherapy.

Peripheral blood was collected in citrate from each subject and the mononuclear cells isolated and purified by a previously described Ficoll gradient procedure [9]. A preparation of 99% pure mononuclear cells was obtained by this method. After at least three washings, these cells were used in the tests described below.

T-lymphocytes

T-lymphocytes were enumerated by the E rosette test using sheep red blood cells (SRBC). Rosettes were defined as lymphocytes surrounded by at least three SRBC. We performed this test in two ways. In the first, or direct test, rosettes resulting from the incubation of mononuclear cells and SRBC alone were counted (ERFC). In the second, or AB serum test, AB human serum, previously decomplemented and absorbed with sheep RBC was added to the incubation mixture (EABRFC). We, as others, have previously shown that this latter procedure gives a higher number of rosettes than the former [10, 11] and we have confirmed this observation in our current work (Table 2). This phenomenon may be related to the detection of the total number of T cells by the sensitized test, while the direct test may define a sub-population of this group.

Table 2. Comparison of rosette formation with and without serum AB

	ERFC		EABRFC	
	(%)	(No.)	(%)	(No.)
Chemotherapy	35	496	42	696
Immunotherapy	32	869	44	1173
Normals	46	1074	56	1299

B-lymphocytes

Enumeration of B-lymphocytes was performed by determination of membrane immunoglobulin (mIg) using a direct immunofluorescent test with a polyvalent fluorescent isothiocyanate conjugated sheep anti-human immunoglobulin serum. Details of this method have been previously described. [9]

Monocytes

Monocytes were enumerated by peroxidase staining as suggested by a recent W.H.O. Workshop [12]. One thousand mononuclear cells were counted for peroxidase positively on each slide.

"Null" cells

After establishing the absolute number of each of the three foregoing groups (EABRFC, mIg + cells, Peroxidase + cells) we calculated the number of so-called "null" cells by the following formula: "Null" cells = mononuclear cells (EABRFC + mIg positive cells + peroxidase positive cells).

Statistical analysis

Statistical analysis were performed by the Student *t*-test.

RESULTS

Table 3 presents a summary of the results of our study and their statistical analysis.

Chemotherapy group

The patients on chemotherapy had a significant reduction in the mean number of mononuclear cells and of the total number of EABRFC in comparison to both the immunotherapy and normal groups. No such difference was noted in either the total number of mIg + or peroxidase + cells. When "null" cells were

Table 3. Summary of results

	Mononuclear cells	EABRFC	mIg Positive	Peroxidase positive	"Null'
Chemotherapy	1470	696	455	344	104 (7%*)
Immunotherapy	2714	1173	467	198	883 (33%*)
Normal	2356	1299	381	396	304 (12%*)
Chemotherapy/ immunotherapy	$P = < 0{\cdot}001$	$P = 0{\cdot}01$	N.S.	N.S.	$P = < 0{\cdot}001$
Normal/chemotherapy	$P = 0{\cdot}02$	$P = 0{\cdot}01$	N.S.	N.S.	N.S.
Normal/immunotherapy	N.S.	N.S.	N.S.	N.S.	$P = < 0{\cdot}01$

*Percent mononuclear cells without markers.

calculated no difference to normal cells was found, but there were far fewer ($P = 0{\cdot}001$) in this group than in the patients receiving immunotherapy.

Immunotherapy group

As discussed above, the immunotherapy patients had a significant elevation of mononuclear cells, EABRFC cells, and "null" cells over the chemotherapy group. Although there were differences in the total number of mononuclear cells and of the subpopulations between the immunotherapy and normal group, the only statistically significant difference was in the calculated "null" cells. There was a much larger number of such cells in the immunotherapy group ($P = 0{\cdot}01$).

DISCUSSION

In our study the percentage and total number of EABRFC, mIg positive, and peroxidase positive cells in normal individuals were in the range previously published [13]. The calculated "null" cells are also in agreement with available data. Our control group was composed of subjects in an older age group than our patients' groups because of the logistic difficulty of obtaining normal paediatric subjects. Several recent reports give conflicting results on the influence of age of the distribution of B and T cells. Weksler and Hutteforth [14], for example, found no change in the absolute number of peripheral lymphocytes or T and B cells with increasing age, and Carosella [15] demonstrated a decreased percentage of T cells occurring somewhere between 46 and 60 yr of age. Since our control group had a mean age (30 yr) considerably younger than this we believe that age difference does not represent a significant problem in the analysis of our results.

The chemotherapy group was marked by a diminution in total mononuclear count. Surprisingly, our data showed that this decrease involved the T rather than B-lymphocytes. Immunoglobulin levels were consistent with our "B cell" results, in that the chemotherapy group did not vary significantly in its IgG, IgA or IgM values compared to the normal group. These findings differ from the report of Sen and Borella [16] who found that T cells were in the normal range while B cells were decreased in patients with acute lymphocytic leukemia on the last day of a 3 year chemotherapy programme. Although the difference in results remains unexplained, the shorter period of chemotherapy in our patients (8–9 months) may in part account for this discrepancy.

A striking finding of our study is the significant elevation in the absolute number and percentage of "null" cells in the immunotherapy group as compared with both the chemotherapy and normal groups. Although the nature of these cells is still unknown, several hypotheses concerning their increase in this situation can be entertained.

1. These cells may represent abnormal elements persisting even during apparently complete remission of acute lymphocytic leukemia. The continued perfect clinical and cytological condition of these patients does not favour this hypothesis.

2. They may be stem cells circulating in the peripheral blood. Although it has been demonstrated that BCG can increase hematopoietic stem cells in mouse bone-marrow [17] human data is lacking.

3. They may be "K" cells. It has been shown that there is increased "K" cell activity in patients on BCG therapy for acute lymphocytic leukemia in comparison to patients receiving no treatment [18]. This hypothesis seems plausible since it has recently been suggested

that "K" cells may be "null" cells [19]. Arguments supporting this hypothesis are now in progress in our laboratory [20]. Lelarge *et al.* unpublished.

4. They may represent T or B lymphocytes or monocytes which have lost any detectable markers, a change possibly induced by immunotherapy.

5. A final possibility is that there may not be a true increase in these cells, but rather a redistribution between the peripheral blood and the various reticulo-endothelial organs.

Further studies are in progress in our laboratory to confirm these preliminary results and to elucidate the nature of these intriguing cells.

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Active Immunotherapy Trials on Acute Lymphoid Leukemia Lymphosarcoma and Acute Myeloid Leukemia

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Abstract—*Active immunotherapy (A.I.) has given 85% cure expectancy to patients of all ages suffering from acute lymphoid types of leukemia called "microlymphoblastic". Cure expectancy for those with the prolymphocytic or macrolymphoblastic types is 40–50%.*

In the case of terminal leukemic lymphosarcoma, patients submitted to the same protocol as acute lymphoid leukemia protocol are surviving for more than 4 years in perfect condition; this has never been achieved with maintenance chemotherapy.

In the case of acute myeloid leukemia, AI prolongs both remission and survival, and recent methods of treatment give grounds for believing that results can be further improved.

Initial results for chronic myeloid leukemia give reason to hope that immunoprevention of the terminal blastic crisis will prove possible.

As regards chronic lymphoid leukemia, the present aim is immunorestoration.

Immunotherapy is extremely well tolerated—treatment was only stopped in one out of 277 cases—whereas long-term maintenance chemotherapy has a high mortality rate for patients in remission.

Immunotherapy appears to prevent the late relapses which seem frequent in patients undergoing maintenance chemotherapy only.

FROM 1963 to the present day we have submitted 300 leukemia patients to active immunotherapy [1–5].

PRINCIPLE OF IMMUNOTHERAPY

As we have shown in several experimentally-induced leukemias [2, 6, 7], immunotherapy is based on the principle of stimulating immune reactions to leukemia associated antigens. This is done either by a specific process—injection of tumour cells sterilized by *in vitro* irradiation—and/or by a non-specific process that consists of applying one or several immunity adjuvants. In our experimental work, we found it more effective to combine both processes rather than use only one.

Immunotherapy is immune treatment applied to the established illness; this distinguishes it from immunoprevention, which is the administration of treatment before tumour establishment. We found that immunotherapy was able to kill all the tumour cells on condition that their number did not exceed 10^5 [6].

This property indicates the place of immunotherapy among available methods of treating leukemia. Since chemotherapy cannot kill the last cell [8] because it obeys first order kinetics [9, 10, 11] and, when effective, only kills *a certain proportion of cells whatever their number*, it is logical to treat leukemia by a sequential combination of chemotherapy and active immunotherapy.

In patients kept in remission by a single "maintenance" course of chemotherapy, lymphocyte DNA has been shown to have the same virochemical marker as leukemia cells during a perceptible phase of the illness [12,13]. Moreover Jean Bernard has shown that patients undergoing only chemotherapy "maintenance" treatment are often subject to late relapses [14]. For both these reasons we feel fully justified in having fixed the duration of immunotherapy at five years, as early as 1963. In fact, immunotherapy might also have an immunopreventive effect on neoplasia reinduction by viral or virus-dependent factors such as DNA [see reference 15].

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It is logical to apply active immunotherapy exclusively to leukemia if tumour antigens are present in the cells. In our laboratory, Doré and Guibout confirmed this hypothesis [16] and showed that it could not only be applied to acute myeloid leukemia, but also to acute lymphoid leukemia (ALL).

MATERIAL AND METHODS

Active immunotherapy stimulation is therefore of two kinds: specific and non-specific.

Specific stimulation. Our method consisted of administering intradermal or subcutaneous injections of $4 \cdot 10^7$ irradiated cells kept at −196°C*, taken out of a blood pool from patients with the same type of neoplasia. For a month this dose was given weekly and thereafter, monthly [17].

Specific stimulation can also be obtained by administering a strain of acute lymphoid leukemia cells in culture* [18].

Non-specific stimulation. As a non-specific immunity adjuvant, we use fresh live BCG from the Pasteur Institute.† The method of administration, based on studies of the dose/effect relationship in Man [19], is as follows: BCG is applied to scarified areas formed by 5 cm long scratches arranged in a square. Within the square, 10 scarifications are made lengthwise with a vaccinating needle, and 10 widthwise, bringing the total length of the scarifications to one metre. Fifty milligrams of BCG are applied to this area, sufficiently slowly to ensure complete penetration. Slight bleeding is not contraindicated, indeed it is preferable.

The number of scarified areas to which BCG is applied depends on the cutaneous reactions to the various antigens (PPD, mumps and Candida). If the reactions are negative, BCG is only applied to one scarified area on one limb, and the limb is changed every week, which means each of the 4 limbs is given BCG every month.

On the other hand, if at least one reaction is positive, four areas, one on each limb, are scarified for each weekly application, and BCG is applied to all four. Consequently, patients with positive reactions are given four times as much BCG as those with negative reactions.

Scarifications were made three times a week for the first three weeks of treatment, twice a week from the fourth to sixth weeks, and once a week from the seventh week onwards.

Other adjuvants, including Corynebacterium parvum and C. Granulosum, and a polynucleotide [Poly I–Poly C] [see reference 2], were used in addition to BCG in treating some groups of patients, but we were unable to show that these combinations were more effective than BCG alone.

Timing of applications. Although active immunotherapy can only kill a limited number of tumour cells, it can kill the last cell. We, therefore, applied it after the following two phases, designed to reduce the cell load:

*These cells may be obtained by fellow practitioners from the ICIG, Hôpital Payl-Brousse, 14–16 avenue Paul Vaillant-Couturier, 94800 Villejuif, France.

†Currently sold under the trade name of Immuno-BCG-Pasteur-F. We have shown that this preparation, stored at 4°C, enables the bacteria to retain their adjuvant action for three months.

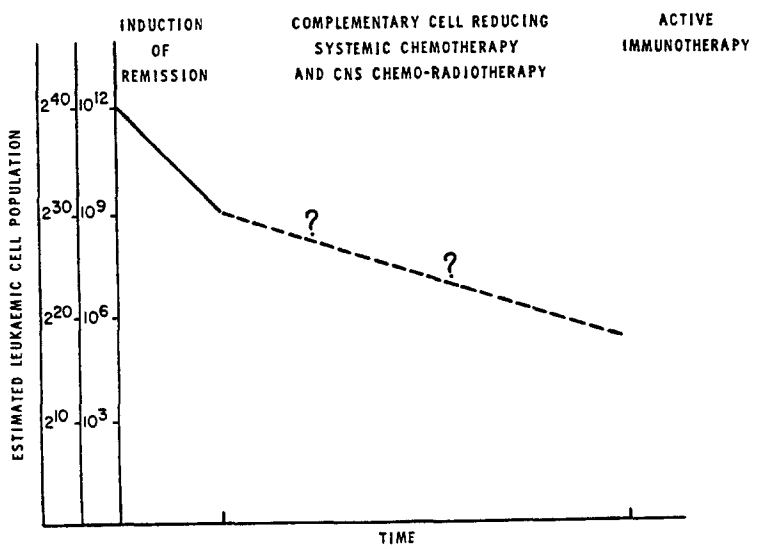

Fig. 1. Combination of remission-inducing sequential chemotherapy, complementary cell-reducing chemo and radiotherapy, and active immunotherapy.

(*a*) the chemotherapy phase, during which remission is obtained and (*b*) the general chemotherapy and complementary cell-reducing meningeal chemoradiotherapy phase [20]. The sequence is illustrated in Fig. 1.

Tolerance. Irradiated leukemia cells were extremely well tolerated. Tolerance of BCG, which we recently assessed in 277 patients, was also remarkably good as we were only obliged to stop administering it in a single case (Table 1).

Table 1. *Comparative toxic cost of active immunotherapy and of "maintenance chemotherapy" in acute lymphoid leukemia treatment*

	Deaths	Severe not lethal toxicity
*Active immunotherapy**		
G. Mathe *et al.* [21]	0/277 patients	
"Maintenance chemotherapy"		
J. V. Simone [22] (Protocol V)	3/31 patients	
J. V. Simone [22] Protocol (VI)	5/94 patients	
J. V. Simone [22] (Protocol VIa)	2/10 patients	
J. V. Simone [22] (Protocol VII)	4/94 patients	
J. V. Simone [22] (Protocol VIII)	2/92 patients 2/17 patients	
R. Aur . . . & J. V. Simone [23]	3/20 patients	6 Crippling Leucoencephalopathy/20
Cl. Jacquillat & J. Bernard [24] (Protocol 06 LA/66)	6/81 children 6/21 adults	
F. Lampert *et al.* [25] (Early irradiation)	4/79 patients	
(Late irradiation)	1/19 patients	
F. Mandelli *et al.* [26]	2/30	
T. S. Gee *et al.* [27]	3/74 children	
A. C. Smith *et al.* [28]	2/17 adults	

*Cure expectancy of the microlymphoblastic type after active immunotherapy: > 80.

The innocuous nature of immunotherapy contrasts sharply with the extremely toxic and particularly lethal effects of "maintenance" chemotherapy, which kills 10 to 20% of patients during remission and sometimes more (Table 1) not to mention the long-term risks it can involve such as carcinogenesis, sterility and genetic changes in descendants [20].

RESULTS

1. *Acute lymphoid leukemia*

1.1. *Initial trial.* In 1963 we conducted a trial with controls, in which we compared the results for 20 randomized patients submitted to chemotherapy, followed by immunotherapy, with those of 10 randomized cases given the same chemotherapy as the first group but no immunotherapy afterwards [1]. Whereas the ten controls all relapsed within 130 days, 7 out of the 20 patients who underwent immunotherapy are still in perfect health, from 7 to 13 years after immunotherapy started (Fig. 2) [3].

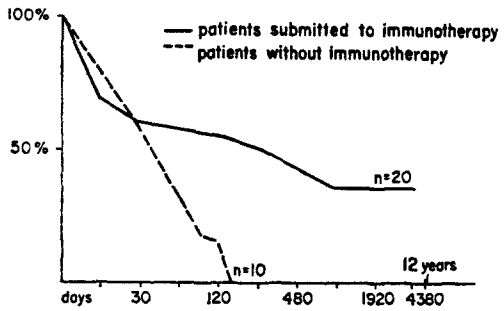

Fig. 2. *Actuarial duration curve for complete remissions in patients undergoing active immunotherapy after cessation of all Chemotherapy (first trial). (Note geometrical time scale.)*

1.2. *Results for the first* 100 *cases.* In 1975 we carried out a retrospective survey of the first 100 patients submitted to immunotherapy whose bone marrow smears were still available for checking diagnosis and establishing their cell type [3, 21].

Their overall cumulative duration of survival while in remission is shown in Fig. 3. It will be noticed that the survival median is over five years, and that in the case of 23 patients, the curve breaks to form a plateau at the 48th month. This plateau is considered as the statistical expression of *cure expectancy.*

Other points worth noting are (*a*) that second remissions after premature relapses

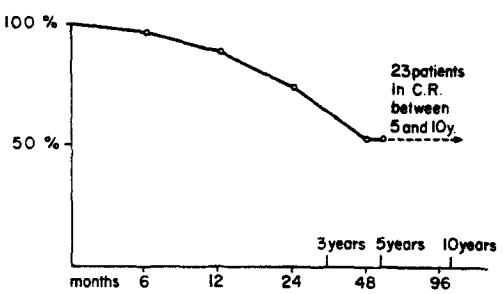

Fig. 3. *Cumulative duration of survival for the first* 100 *ALL patients, in perfect clinical and hematological condition, who underwent active immunotherapy (note geometrical time scale).*

occur with the same frequency (93%) as first remissions [29], and (*b*) that we did not observe any relapses between the fourth and 13th years [3].

Results for immunotherapy are thus in sharp contrast to those for maintenance chemotherapy, for, according to Jean Bernard [14], many patients undergoing the latter treatment have relapses after the fourth year.

1.3. *Confirmation of the effect of immunotherapy on acute lymphoid leukemia.* At first, an MRC group [30] who used Glaxo BCG, which we did not find effective in our experiments [31], was unable to observe the effects of immunotherapy on ALL. They were confirmed, however, by the Hemopathy Working Party of the European Organization for Research in the Treatment of Cancer [32] and by Ekert and Jose in Australia [33].

1.4. *Immunosensitivity and prognosis factors.* All ALLs do not have the same cure expectancy as the one shown in Fig. 3. The factors determining it [2, 3] are not the same as the prognosis factors for patients undergoing a single course of maintenance chemotherapy [24 and 34].

For instance age, which has some bearing in the latter case, makes no difference in immunotherapy.

On the other hand, the size and deposits of neoplastic formations, and more particularly their cell types (Fig. 4) [35, 36], affect our patients and we have been able to show that this last characteristic governs all the others.

Figure 5 shows the different prognoses according to cell type. For patients of all ages with *microlymphoblastic* leukemia, cure expectancy is 85% and for those with the *prolymphocytic* and *macrolymphoblastic* varieties of the disease, it is between 40 and 50%. There is no plateau for those with what is known as the *prolymphoblastic type*, which English-speaking authors call *undifferentiated* and usually discount from ALL statistics.*

It is interesting to observe from Fig. 5 that prognosis based on the cell type is determined by sensitivity to immunotherapy and that such prognosis does not therefore appear in the results Jean Bernard obtained [24, 34] with the patients he submitted to maintenance chemotherapy alone.

Our findings with regard to prognosis factors led us to adapt our protocol to them.

*The 100 cases we studied did not include any patients with the type of leukemia recently described as the acute immunoblastic variety [37].

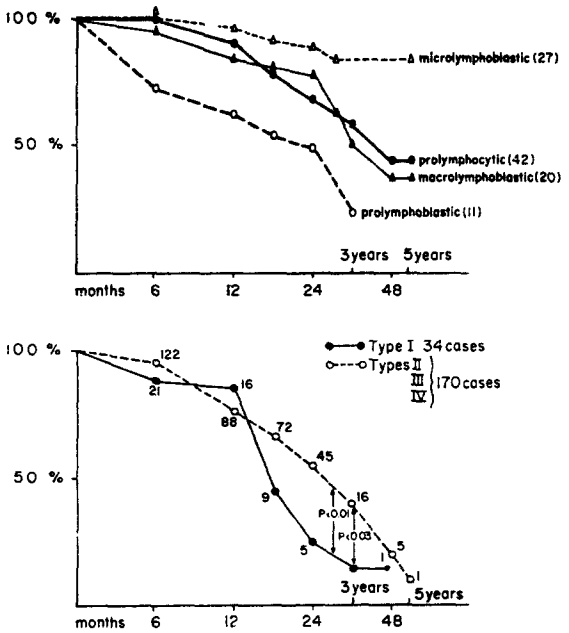

Fig. 5. Upper part: Cumulative duration of survival in patients undergoing active immunotherapy according to the following cell types:

(*a*) *microlymphoblastic*
(*b*) *prolymphocytic*
(*c*) *macrolymphoblastic*
(*d*) *prolymphoblastic (or undifferentiated?)*

The first three types only form a plateau after five years. Lower part: Cumulative duration of survival, for the same cell types, in patients submitted to maintenance chemotherapy by J. Bernard et al. [24, 34]; *This difference in prognosis does not recur in maintenance chemotherapy patients treated by J. Bernard* et al. *It is therefore reasonable to suppose that the difference found in our patients, depending on their cell type, is determined by their respective sensitivity to immunotherapy. (Note that time scale is geometrical for these curves.)*

Figure 6 shows that protocol 12 includes pre-immunotherapy cell-reducing chemotherapy which is more intensive for the cases with the least favourable prognosis.

With regard to our trials, the use of another immunity adjuvant in addition to BCG—in our case Corynebacterium parvum, C.granulosum* or Poly I–Poly C—did not improve the results for BCG [2].

2.

For *leukemic terminal lymphosarcoma* [5] a few patients were treated by active immunotherapy, using a combination of irradiated tumour cells, BCG and C. granulosum. The results were better than those for maintenance chemotherapy (Fig. 7) and five of these patients are today in perfect health, 13 to 66 months after the start of treatment.

*This adjuvant should only be used in extremely specialized centres where the different immune-functions can be measured as it inhibits the T-dependent reactions essential to antitumoral immunity [2].

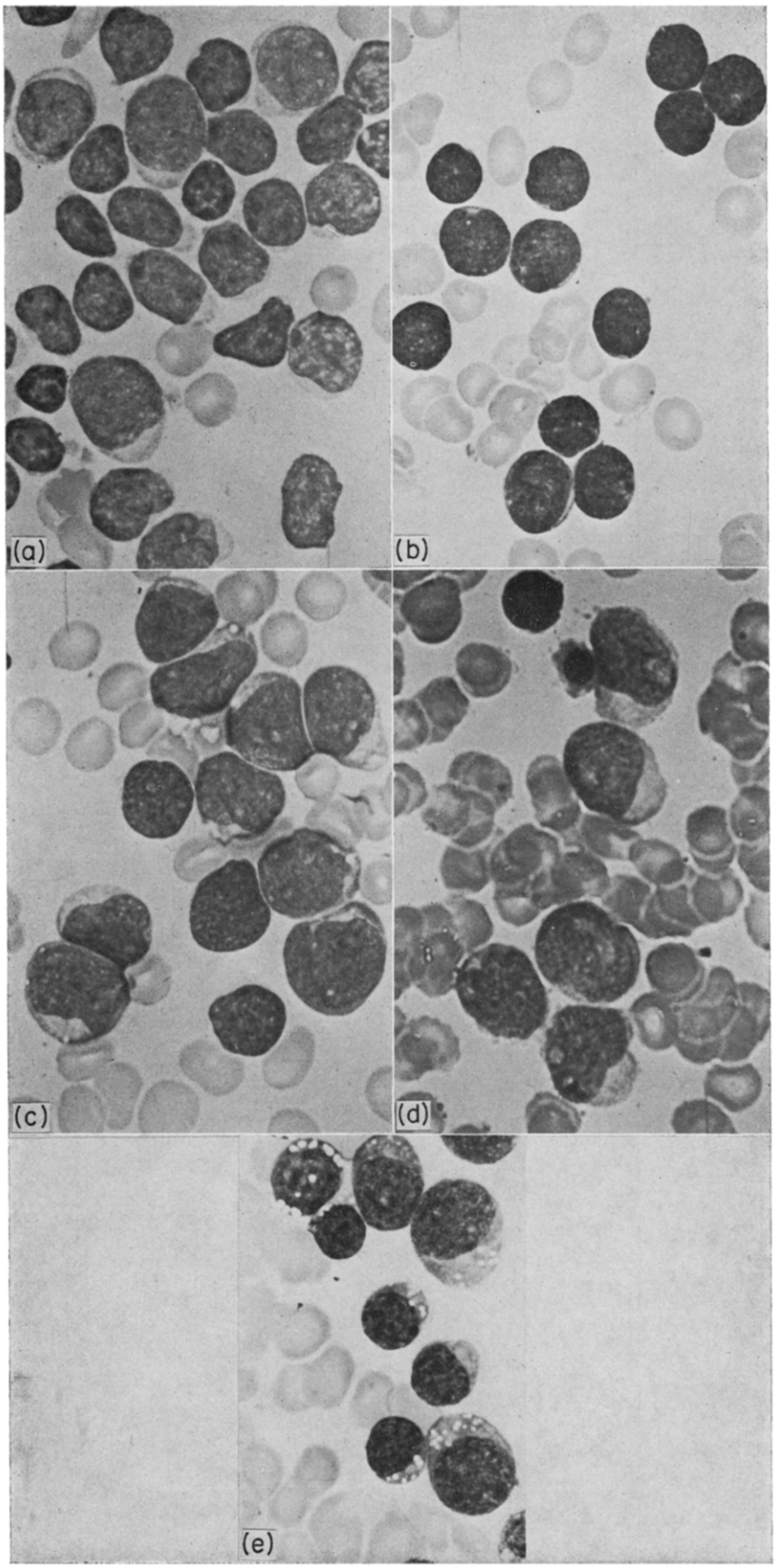

Fig. 4. *The five types of acute lymphoid leukemia: (a) microlymphoblastic, (b) prolymphocytic, (c) macrolymphoblastic, (d) prolymphoblastic, (or undifferentiated?), (e) immunoblastic* [*see* 36 & 37]. *The* 100 *patients studied do not include cases of the immunoblastic type* [38].

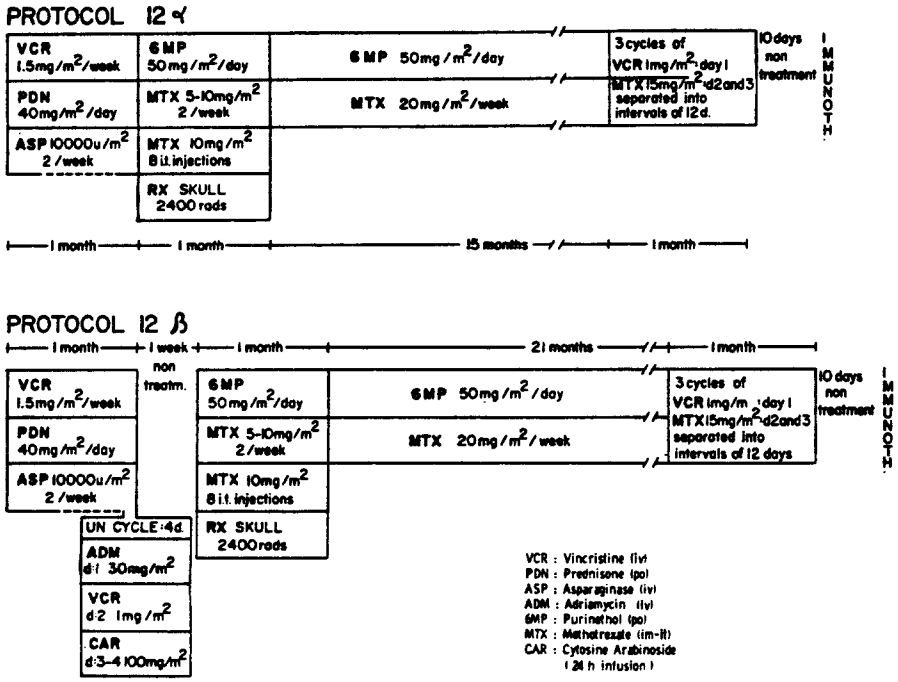

Fig. 6. Protocol 12 currently applied to patients suffering from acute lymphoid leukemia or leukemic lymphosarcoma. α type: patients with signs of a good prognosis, β type: patients with signs of a poorer prognosis. After remission induction, the latter type is given a complementary course of treatment called a reinforcement course, as well as a longer course of cell-reducing chemotherapy than that included in the α type protocol.

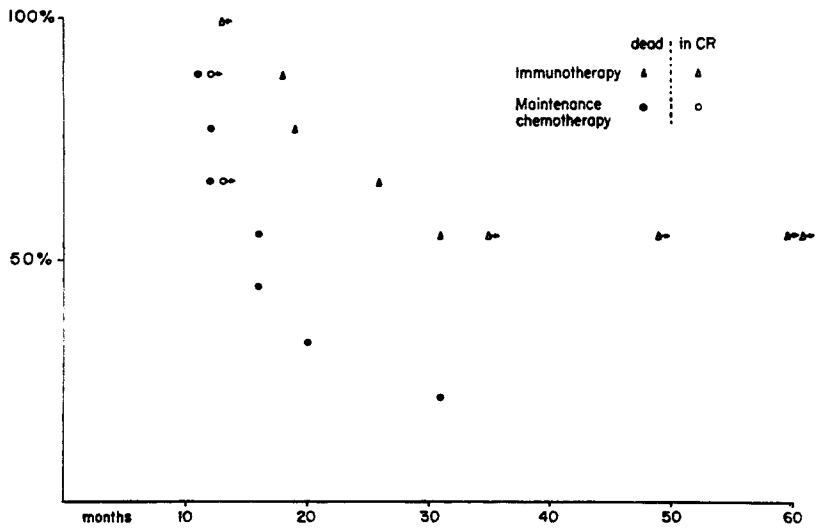

Fig. 7. Comparison of cumulative survival in two groups of patients with terminal leukemic lymphosarcoma. One group followed the same protocol as the ALL cases, including immunotherapy, and the other was treated by maintenance chemotherapy.

3.

In the case of *acute myeloid leukemia*, the results of several trials have been published. They confirm the effects of active immunotherapy using either BCG alone [39, 40] or neuraminidase-treated tumoral cells alone [41], or, again, a combination of BCG and neoplastic cells [4, 42]. The results of our own trials were favourable (Table 2 and Figs. 8 and 9), but less satisfactory than those for ALL or for the trial in which neuraminadase-treated cells were used [41].

The protocol we are applying at present is

Table 2. Acute myeloid leukemia active immunotherapy

		Medians	
		of first complete remissions (FCR)	of survival after FCR
Maintenance chemotherapy	EORTC first trial [43]	32 weeks	55 weeks
	EORTC second trial [44]	34 weeks	N.D.
	EORTC Villejuif patients in both trials	26 weeks	52 weeks
	Vogler and Chan [39]	26 weeks	
	Powles *et al.* [42]	26 weeks	46 weeks
	Gutterman *et al.* [40]	60 weeks	
Chemotherapy interspersed with immunotherapy	Gutterman *et al.* [40]	72 weeks	
	Powles *et al.* [42]	58 weeks	77 weeks
Chemotherapy followed by immunotherapy	Powles *et al.* [42]	48 weeks	83 weeks
	Vogler and Chan [39]	39·4 weeks	
	Mathe *et al.* [4]	60 weeks	104 weeks

N.D. = not determined.

therefore designed to study the use of cells treated by the latter enzyme.*

4. *Chronic myeloid leukemia (CML) and chronic lymphoid leukemia (CLL)*

For CML, Sokal's results [46] are very encouraging. As with his treatment, our present protocol aims at immunoprevention of the blastic crisis.

As regards CLL, our current trial is designed to restore immunity, which is often insufficient [2].

*Doctors may obtain these cells from the Institut de Cancerologie et d'Immunogénétique, Hôpital Paul Brousse, 14–16 avenue Paul-Vaillant Couturier, 94800 Villejuif.

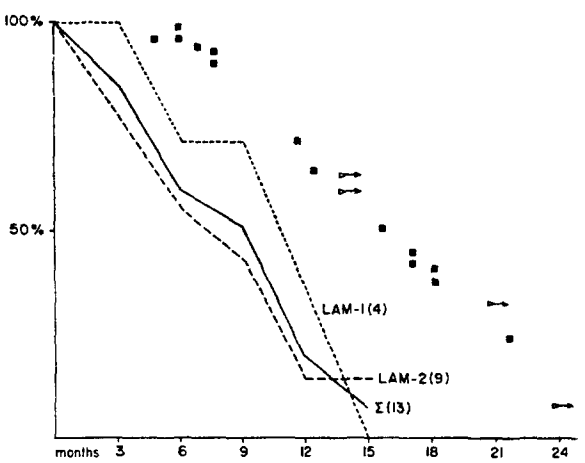

Fig. 8. Comparison of cumulative remission in acute myeloid leukemia patients undergoing treatment including active immunotherapy (irradiated leukemia cells + BCG) with patients we submitted to a European protocol, comprising maintenance chemotherapy only without immunotherapy.

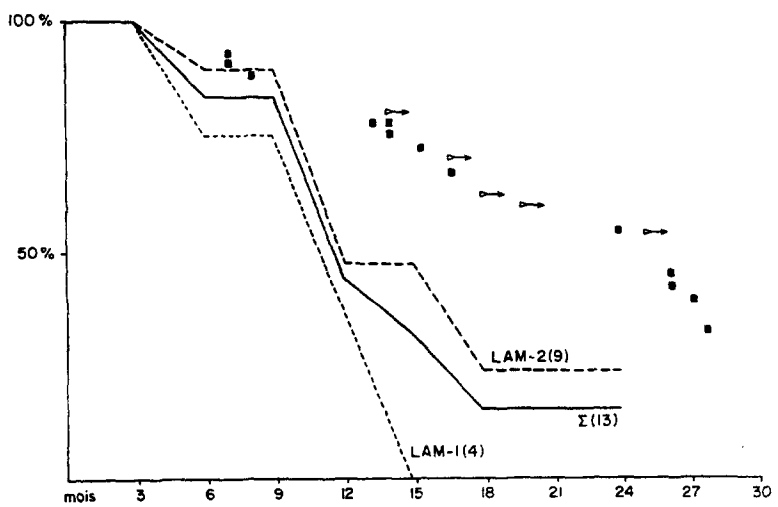

Fig. 9. Comparison of cumulative survival in acute myeloid leukemia patients treated by active immunotherapy (irradiated leukemia cells + BCG) with patients we submitted to a European protocol comprising maintenance chemotherapy only without immunotherapy.

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Splenic and Hassall's Corpuscles Modifications Following Injection of Fresh BCG in the Guinea-Pig

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Abstract—*The results indicated that i.v. injection of fresh BCG induced in the guinea-pig thymus a transient increase of lymphocyte mitosis associated with an epithelial hyperplasia in the cortex on day 10. Changes in the medulla were more obvious on day 15. The hyperplasia of Hassall's corpuscles and epithelial cells in the medulla reached its maximum level at a time when many lymphocyte masses appeared in the splenic red pulp and in the T area of the spleen. Evidence suggesting an active secretory function in their external cells was recorded. These findings were consistent with the hypothesis that BCG produces an increase of new lymphocytes input into the T lymphocyte cycle.*

INTRODUCTION

IT HAS been suggested [1] that Hassall's corpuscles were not only a site of lymphoid cells destruction, but were also a privileged area for maturation of medullary lymphocytes, at least in the foetal guinea-pig thymus. Since it has been shown that BCG injections induced a transient proliferation of lymphocytes in the mouse thymus [2], it was interesting to ascertain if Hassall's corpuscles would also be involved in this process. In this regard, Hassall's corpuscle changes might be a marker visualizing a modification of the lymphocyte cellular kinetics in the thymus.

In the present work, both electron and light microscopic and quantitative histological techniques were used to study the development of Hassall's corpuscles and to achieve information on their relationship between thymic and splenic changes during the process of immunization by BCG.

MATERIAL AND METHODS

Experimental animals

One hundred and eight male and female guinea-pigs (3–4 months old) were used. They were randomized into 8 experimental groups and 1 control group. Each group consisted of 12 guinea-pigs.

Treatment

Pasteur Institute BCG was employed. It comprises 7×10^6 viable units per mg. It was a fresh non-lyophilised preparation less than 7 days old. Each guinea-pig was injected with 12 mg of BCG. Intravenous (i.v.), subcutaneous (s.c.), intraperitoneal (i.p.), and intradermal (i.d.) routes of administration were employed. The guinea-pigs were injected on 10th and 15th day before they were sacrificed.

Tissue preparation

The thymus and the spleen were rapidly removed and weighed on electric balance. For light microscopic studies, whole thymic lobes were fixed in Bouin-Holland solution. Twenty-eight to 51 serial sections were cut for each lobe.

These sections, approximately 5 μm thick, were stained with Hematoxylin-Eosin, Methyl-green Pyronin and P.A.S. The spleen was treated in a similar fashion. For quantitative histological studies, at least 28–51 serial sections were micrographied for each thymus. Surface measurements of Hassall's corpuscles and medulla were made on micrographs enlarged photographically to a final magnification of 350 ×. A transparent plastic sheet ruled with a square grid containing 25 squares was placed over each micrograph to subdivide it

into areas for counting. Each square represented an area of 812 μm^2 on the original section. To express the results the index "I" was determined by the expression:

$$I = \frac{\text{Mean Surface Area of Hassall's Corpuscles}}{\text{Mean Surface Area of Medulla}} \times 100$$

The Student *t*-test was employed to statistically evaluate the results.

For electron microscopic studies small pieces of tissue were fixed according to Sabatini *et al.* [3] and embedded in epon [4]. Ultra thin sections were cut with a glass knife on a L.K.B. ultramicrotome, mounted on 200 mesh uncoated grids and doubly stained with saturated uranyl acetate and lead citrate [5]. The grids were examined with a J.E.M. 100 C electron microscope (Jeol).

RESULTS

Macroscopic observations

There was no significant difference of thymic weight in all experimental groups. Significant differences in the splenic weight were noted on day 10 in i.v. and i.p. injected guinea-pigs. On day 15, significant differences were recorded in i.v., s.c. and i.p. injected guinea-pigs, but not in the i.d. injected group. A striking difference in the splenic weight occurred between day 10 and day 15.

Microscopic observations

The thymus: day 10. In the cortex the thymic sections of the i.v. injected guinea-pigs showed an increase of the mitotic activity of lymphocytes associated with an epithelial hyperplasia (Fig. 1).

In the medulla an epithelial hyperplasia was also recorded. Large epithelial cells were gathered in clusters (Fig. 2). Under electronic examination the largest sized cells appeared engaged in a keratinizing process (Fig. 3).

Day 15. The cortical reactions were less obvious or disappeared, but in the medulla the epithelial hyperplasia was more conspicuous. Numerous single epithelial cells resembling Mandel cystic cell [6] were observed (Fig. 4). A great amount of small full Hassall's corpuscles were noted (Fig. 5). Cavity corpuscles were readily invaded by granulocytes. Many degenerating corpuscles were equally observed. The external cells [1] constructing the peripheral zone of the Hassall's corpuscles contained an appreciable number of dilated ergastoplasmic cisternae vacuoles (Figs. 5 and 6). These vacuoles varied in size and sometimes exhibited an amorphous substance having the appearance of secretion (Fig. 7). The wall of the largest vacuoles was provided with short microvilli which protruded into the lumen (Fig. 8). Dense granules similar to glycogen particles were conspicuous in their cytoplasm (Fig. 8). Large and medium sized lymphocytes were located in intimate contact with the external cells (Figs. 5 and 6).

The "I" index changes confirmed (Table 1) that the volume of Hassall's corpuscles increased on day 20 in i.v. injected guinea-pigs. The increase was more striking on day 15 in i.v., s.c. and i.p. injected groups.

Table 1. *This table shows that the volume of Hassall's corpuscles increased on day* 10 *in i.v. injected guinea-pigs. These results were confirmed on day* 15 *in i.v., s.c. and i.p. injected groups*

Days	Routes	I. Index
10[c]	i.v.	11·11 ± 1·21 S.[b]
	s.c.	10·63 ± 3·23 N.S.
	i.p.	8·48 ± 1·91 N.S.
	i.d.	8·71 ± 2·1 N.S.
15	i.v.	13·69 ± 1·30 S.
	s.c.	12·26 ± 1·47 S.
	i.p.	11·54 ± 1·81 S.
	i.d.	9·67 ± 1·81 N.S.
Control		8·36 ± 1·69[a]

$$\text{I index} = \frac{\text{Mean Surface of Hassall's Corpuscles}}{\text{Mean Surface of Medulla}} \times 100$$

(a) Mean ± Standard Error.
(b) Student *t*-test
S.: Significant.
N.S.: Non Significant.
(c) Days after injection.

The spleen: day 10. The splenic sections of the i.v. injected guinea-pigs depicted a considerable amount of multiple granulomas in the red pulp cords. They consisted of epithelioid and giant cells (Figs. 9 and 11). The red pulp was enlarged and multiple lymphocytic foci were seen distinctly outside the Malpighian corpuscle (Fig. 9).

Day 15. The splenic red pulp was considerably enlarged. The lymphocytic infiltration also involved the periarteriolar zone of the white pulp (Fig. 11). In s.c. and i.p. injected guinea-pigs these changes were less obvious. They were very attenuated in the i.d. injected group.

DISCUSSION

The results indicated that i.v. injection of

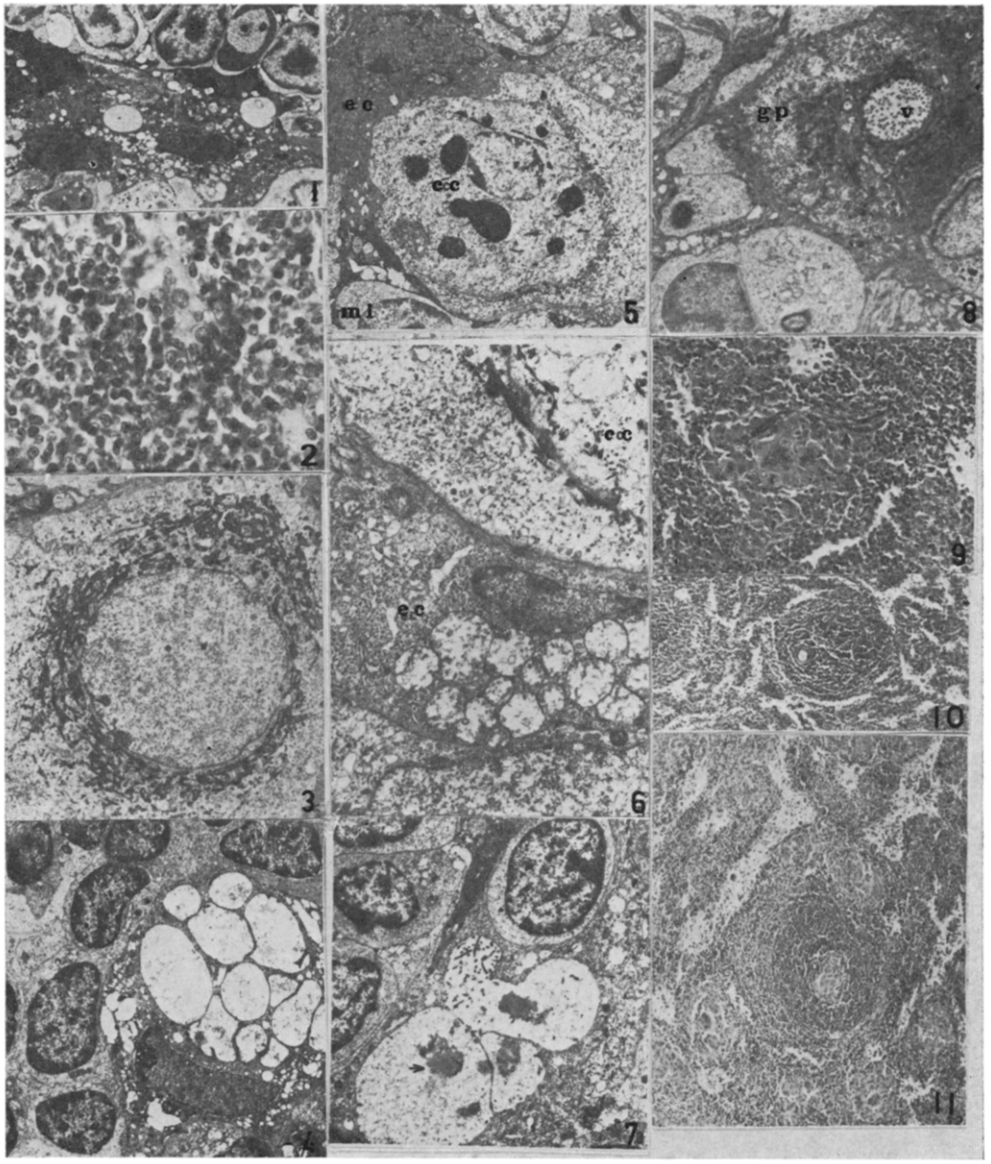

Fig. 1. *Day* 10 *i.v. injected guinea-pig. Epithelial hyperplasia in the cortex* × 1500.
Fig. 2. *Day* 10 *i.v. injected guinea-pig. Epithelial hyperplasia in the medulla H.E.* × 185.
Fig. 3. *Day* 10 *i.v. injected guinea-pig. Cell engaged in a keratinizing process* × 2700.
Fig. 4. *Day* 15 *i.v. injected guinea-pig. Single cystic epithelial cell in the medulla* × 1800.
Fig. 5. *Day* 15 *i.v. injected guinea-pig. Full Hassall's corpuscle* × 1850.
e c: external cell.
m l: medium sized lymphocyte.
Fig. 6. *Day* 15 *i.v. injected guinea-pig. Full Hassall's corpuscle* × 4550.
e c: external cell.
Fig. 7. *Day* 15 *i.v. injected guinea-pig. External cell: vacuoles containing an amorphous substance* × 2350.
Fig. 8. *Day* 15 *i.v. injected guinea-pig. External cell* × 1050.
g p: glycogen particles.
v: cystic vacuoles.
Fig. 9. *Day* 15 *i.v. injected guinea-pig. Granuloma and lymphocytic foci in the red pulp H.E.* × 100.
Fig. 10. *Control: a Malpighian corpuscle H.E.* × 50.
Fig. 11. *Day* 15 *i.v. injected guinea-pig: an enlarged Malpighian corpuscle H.E.* × 50.

fresh BCG induced in the guinea-pig thymus a transient increase of mitosis and an epithelial hyperplasia in the cortex on day 20. Changes in the medulla were more obvious on day 15. In this area, some epithelial cells were isolated and were similar to cystic cells descirbed by Mandel [6]. Numerous epithelial cells were gathered in clusters identified as new Hassall's corpuscles [1]. Evidences suggesting an active secretory function were obvious in their external cells. Since epithelial cells have been proposed as producing thymic factor(s) involved in immune function [7–13], it is worthy to note that external cells were in close contact with large and medium lymphocytes. The "I" index changes suggested a significant increase in the relative volume of Hassall's corpuscles. The hyperplasia of Hassall's corpuscles and epithelial cells in the medulla reached its maximum level at a time when many lymphocytes masses appeared in the splenic red pulp and in the T-area of the spleen. These findings were consistent with the hypothesis that BCG acts in cancer immunoprophylaxis or immunotherapy [14–24] in increasing the input of many lymphocytes into the T lymphocyte cycle [2, 22]. On a theoretical level, this work offers new evidence of Hassall's corpuscle involvement in certain immunological processes. On a practical level, this paper delineates the importance of BCG route.

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Clinical Trial of Poly I – Poly C as an Immunity Adjuvant and an Immunorestoration Agent

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Abstract—*We present preliminary results in cancer patients of an attempt at non-specific cell-mediated immunorestoration with poly I – poly C during a treatment of 28 days.*

Two out of 9 patients have shown decreased responses to recall antigens, while the in vivo immune status of 7 out of 9 remained unchanged.

Two out of 9 non-responders to PHA, 2 out of 8 non-responders to PPD, and 4 out of 8 non-responders to an allogeneic lymphocyte population were converted to responders.

Two out of 5 patients without any LIF activity before, presented positive inhibition after the treatment.

Numeration by surface markers of T, B and monocytes gave no noticeable change.

We suggest that null or negative effects on DHR are in keeping with the load of the tumour.

Our in vitro results confirm previously published findings on short-term leucocyte cultures with mitogens and antigens.

INTRODUCTION

SYNTHETIC polynucleotide complexes have been demonstrated as antibacterial and antiviral agents [1–3], and also as adjuvants of immunity by improving immunological competency, as has been widely shown in experimental models [4–7].

Poly A – poly U and poly I – poly C have been found to increase macrophage activity as well as T and B cell responses [8–10].

The capacity of synthetic polynucleotides to enhance both limbs of the immune response, and to increase host resistance against tumours led us to conduct a clinical trial in patients with solid tumours in an attempt to restore or stimulate their immunocapacity.

We will present here preliminary results from the immunological examination of nine cancer patients and on possible changes in their immune status by treating them with a synthetic copolymer of poly-inosinic and poly-cytidylic acids (poly I – poly C) for 28 days.

MATERIAL AND METHODS

1. *Patients*

Six males and 3 females were included in this trial; 4 patients with melanoma, 1 patient with lung carcinoma, 1 patient with gastro-intestinal tract carcinoma and 2 patients with Hodgkin's disease; mean age was 53 yr (30–69).

2. *Preparation of poly I – poly C*

One milligram of a mixture of homopolymer poly I and poly C, and one sample of solvant (Choay, France) are left for 30 min at 37°C in a water bath. One dose of poly I – poly C is then diluted with solvent, and the required dose or doses are added to 125 ml of saline. The product is rapidly administered by i.v. injection.

3. *Dosage and time schedule of administration of poly I – poly C*

Details of administration are listed on Table 1.

Table 1. Dosage and time schedule of administration of poly I – poly C

Length of treatment	= 28 days
Dosage an time schedule	= days 1–3 1 mg days 4–6 2 mg days 7–9 3 mg days 10–28 4 mg
Route of administration	= i.v. injection in 125 ml of saline
In vivo tests	= at days: −1, +15 and +35
In vitro tests	= at days: −1 and +35

Table 2. Screening for an assay of immuno-restoration non specific immuno-exploration

In vivo tests: Skin tests	Recall antigen Protein purified derivative (PPD) (10 U) Mumps (2 U) Candida (1/10000) Streptokinase (1/100)
In vitro tests: Lymphocyte and monocyte counts (per mm^3 of blood) Lymphocyte transformation by: Phytohemagglutinin Pokeweed mitogen PPD Mixed allogeneic lymphocyte reaction (MLR)	Immunoglobulins Electrophoresis and immunodiffusion Percentage of mononuclear cells (a) Forming spontaneous rosettes with sheep erythrocytes (b) Carrying surface immunoglobulins (direct fluorescence) (c) Peroxydase positive cells (d) "Null" cells LIF activity in serum

4. *Surveillance of patients*

All patients are submitted to a non-specific immunological examination: exploration of *in vivo* and *in vitro* reactions. Tests are listed on Table 2.

(*a*) Delayed hypersensitivity reactions to recall antigens are estimated in mm of induration by a double blind estimation. (A positive reaction is an induration of > 5 mm).

(*b*) Lymphoblastic transformation tests by mitogens and antigens have previously been described [11] according to the method of Bréard [12].

(*c*) Numeration of subpopulations of mononuclear cells are tested by surface markers according to the techniques described by Belpomme [13].

(*d*) Leucocyte migration inhibiting factor (LIF) activity in serum is evaluated by a micromethod, according to the technique of Beaulieu (personal communication) which has been previously described [14].

RESULTS

1. *Toxicity*

No manifestation of toxicity was observed in this preliminary trial.

2. *Immunological effect*

2.1. *Delayed hypersensitivity reactions (DHR).* All patients were tested with four recall antigens.

We found no change in DHR after 15 days of treatment. Four out of 4 patients were anergic and remained at that time as non-immunoresponders *in vivo*. Among allergic patients, 3 patients presented one positive reaction with an induration of 10 mm, 1 patient had 2 positive reactions, and one

Table 3. Effect of administration of poly I – poly C on in vivo *and* in vitro *tests*

	On skin tests*		On lymphocyte transformation†			On LIF activity in the patient's serum‡
			PHA	PPD	MLC	
	d+15	d+35	d+35			d+35
Number	9		9	8	8	5
Conversion to positive reactions	0	0	2	2	4	2
Unchanged reactions	9	7	7	5	4	3
Decreased reactions	0	2	0	1	0	0

*Delayed hypersensitivity reactions to recall antigens (PPD, Mumps, candida and streptokinase): A patient is called allergic if he presents at least one positive reaction to a recall antigen with an induration > 5 mm.

†Lymphocyte transformation by PHA: A patient is said to respond to PHA if the incorporation of ^{3}H-thymidine after 4 days incubation is at least of 18·000 counts/min (according to the distribution obtained with 80 normal adult volunteers). By MLC and PPD if the stimulation index is > 5 × the background.

‡LIF activity is present if patient's serum inhibit normal leukocyte migration of at least 20% compared to normal serum.

patient 3 positive reactions. These allergic patients had no variation in their skin reactions at day 15.

But skin tests evaluated one week after the end of the treatment, i.e., at day 35, did not present the same pattern of reactions.

Two patients, 1 with 3 positive reactions and 1 with 2 positive reactions became anergic (Table 3).

2.2. *Analysis of* in vitro *lymphocyte transformation tests.* Critical analysis of these tests in evaluating cell-mediated immunity have been discussed previously [15].

Mitogen responses of lymphocytes are expressed in counts/min. Mean geometric values for PHA and PWM in a control population were respectively 70,000 counts/min and 25,000 counts/min. Confidence intervals were between 18,000 counts/min and 250,000 counts/min for PHA, and between 6500 and 150,000 counts/min for PWM.

Patients with lymphocyte responses less than 18,000 counts/min for PHA and 6500 counts/min for PWM were called non-responders. Values above these limits were considered as normal values.

Five patients presented normal values by PHA transformation before and after treatment. The mean value was 54,805 ± 4876 counts/min before treatment and 69,141 ± 9363 counts/min after poly I – poly C administration.

Two patients with values of ^{3}H-thymidine lymphocyte uptake of 615 ± 78 counts/min and 5566 ± 1057 counts/min before treatment, presented normal incorporation after treatment, with values of 26,914 ± 1648 counts/min and 137,226 ± 6740 counts/min respectively.

Two non-responsive patients remained with the same pattern of abnormal lymphocyte incorporation (181 ± 13 counts/min and 146 ± 19 counts/min before treatment, and 17,630 ± 2846 and 17,520 ± 2066 counts/min respectively after treatment).

Antigen responses of lymphocytes were expressed as a stimulation index (S.I. = counts/min in sensitized lymphocytes / counts / min in lymphocytes with medium alone × 100.) Patients with a S.I. 5 are called non-responders to antigens, i.e. PPD and allogeneic lymphocytes.

Responses of lymphocytes to PPD have been analysed.

Two patients had an increased S.I. after treatment (S.I. were of one and three before treatment and of nineteen and fifteen after treatment).

One patient presented a decreased reaction with a S.I. of 10 before poly I – poly *C* and of one after poly I – poly C.

The 5 other patients studied did not present any change in their responses. All had S.I. of less than 5 and remained non-responders to PPD.

Responses in mixed lymphocyte reactions (MLR) have been analysed.

Two patients had an increased S.I. after treatment (S.I. were of 1 and 3 before treatment and of 19 and 15 after treatment).

Four patients with a low S.I. at the beginning of the treatment have shown increased responses at day 35 (3 of them were totally non-responders and one presented a S.I. of 4 before poly I – poly C); S.I. after treatment have been respectively 17, 35, 75 and 68).

Results are summarized on Table 3.

2.3. *Analysis of* in vitro *LIF activity in the serum.* The activity of leucocyte inhibiting factor (LIF) in the serum was considered as positive if the percentage of inhibition of normal leucocytes was at least 20%. All sera were freshly tested.

Five sera have been evaluated before and after the administration of poly I – poly C.

All sera drawn before the treatment presented no significant inhibition.

Two out of 5 showed a positive inhibition when these sera were taken at day 35. The percentages of inhibition were 20% and 25%.

We did not observe any change in the DHR of these two patients.

Results are summarized on Table 3.

2.4. *The numeration of subpopulations of mononuclear cells.* This has been evaluated by surface marker tests. Results were expressed in absolute numbers of the different populations, and a 25% change more or less in the initial value was considered as a significant change.

T cell numerations have been expressed according to two techniques, with and without AB serum:

(*a*) without AB serum: 6 out of 7 patients showed no significant change, 1 patient presented a significant decrease in this test;

(*b*) with AB serum: 4 out of 7 patients had no significant change; again 1 patient presented a significant decrease, as with the technique without AB serum; and 2 patients, who had shown no change in previous test, had a significant increase in this test: one of these patients presented a significant increase in PHA response, while remaining a non-responder in PPD and MLC tests; the other patient presented no depressed PHA transformation before treatment but no PPD and MLC responses.

This patient has been converted to positive reactions with these antigens after treatment.

B cells numeration by polyvalent serum presented no significant change.

Peroxydase positive cells, i.e., *monocytes*, have shown no significant variation.

2.5. *Correlation between skin reaction tests and* in vitro *lymphocyte transformation by PHA.* Table 4 indicates what had already been observed in a previous study on the effect of BCG on immunodepressed cancer patients [11], that the correlation between *in vivo* DHR and *in vitro* transformation by PHA is only partial [11].

Seven out of 18 tests estimated before and after treatment by poly I – poly C, are correlated. Thus the percentage of correlation between both tests is only 39.

DISCUSSION

Analysis of *in vitro* lymphocyte transformation tests indicated that poly I – poly C might enhance tritiated thymidine incorporation in lymphocytes both with PHA and with antigens. The sensitized lymphocyte response of one patient was depressed. These findings are in accordance with the results of a preliminary report from the Oettgen's group who found, in patients with advanced cancer, 1 enhanced response and 4 depressed responses to PHA[16]. Our results also confirmed the data published by others studying the effect of polynucleotides on short term leucocyte culture, although they systematically found a depressive effect with PHA stimulation, while an enhancing one in sensitized lymphocytes [17, 18].

LIF activity was found to be positive in 2 patients, without any change in their *in vivo* responses. One of them became responsive in *in vitro* transformation tests by PHA and by MLC.

The weak variations in mononuclear cells numeration have been difficult to interpret and it seems possible that for a significant change to appear after a trial of immunotherapy more time is needed; as recently Belpomme *et al.* [19] found no significant change with a 15 days treatment by BCG, while after a long-term immunotherapy they found a significant increase in null cells.

Table 4. Absence of correlation between skin reaction tests in vitro *lymphocyte transformation by PHA*

In vivo tests†	*In vitro* transformation test*	
	Non responders = 9 patients	Responders = 9 patients
Anergic = 10 patients	4	6
Allergic = 8 patients	5	3

*Lymphocyte transformation test by PHA.
†Delayed hypersensitivity reaction to recall antigens (PPD, mumps, candidine, streptokinase).

It has been shown that host resistance against tumours is increased, especially when the tumour load is low as has been demonstrated in patients with acute lymphoblastic leukaemia [20], or when the tumour mass has been diminished by surgery [21]. Thus we postulated that the negative effect in restoring or stimulating DHR in our patients is in keeping with the tumour volume. We cannot however interpret the two depressed *in vivo* reactions found while these patients failed to present any decreased *in vitro* responses.

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Levamisole and Human Lymphocyte Cultures*

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Abstract—*Blood lymphocytes from normal subjects were cultured with levamisole in the absence or presence of phytohemagglutinin (PHA). Levamisole, by itself, did not stimulate the lymphocytes. Levamisole, at 10^{-3} mg/100 ml, significantly increased the response to the mitogen. Other concentrations (from 1 mg to 10^{-8} mg/100 ml) did not alter the response to PHA. The same effect, at the same concentration, was observed for isolated blood T cell rich populations in presence of PHA. Levamisole did not modify the PHA response of T cell poor populations. These experiments suggest that levamisole has an action on human T lymphocytes.*

INTRODUCTION

LEVAMISOLE, an antihelmenthic drug, has recently been much investigated in immunology. This interest lies in the reports that it can stimulate, immune mechanisms in mice and in man [1, 2]. In mice, levamisole has thus been shown to possess stimulatory activity on both humoral and cellular immune responses, when injected into animals at the same time as the antigenic stimulus [1]. Both lymphocytes and macrophages appear to be involved in the mechanisms of levamisole. In man, the drug restores the delayed hypersensitivity responses in the anergic old normal subjects or cancer patients [2]. Little is known regarding the, *in vitro*, effect of levamisole on human lymphocytes. Therefore, we decided to investigate if levamisole can directly stimulate human lymphocytes or influence their response to phytohemagglutinin (PHA), a rather specific T cell mitogen.

MATERIAL AND METHODS

Human lymphocytes were isolated from heparinized blood of six normal volunteers, on a Ficoll–Hypaque gradient. They were washed three times with a Hank's buffered salt solution and resuspended at a final concentration of 20×10^6 cells per ml of medium RPMI 1640 with HEPES (Gibco-Biocult). A fraction of these cells, further referred as unseparated population was used for these cultures [3]. The other fraction was further processed in order to obtain a T cell rich population and a T cell poor population according a rosetting technique previously described [4]. This technique is based on the property of human T lymphocytes to bind to sheep red blood cells (SRBC) in a rosette formation [5]. The SRBC present in the pellet of rosettes (T cells) were lysed with an isotonic solution of NH_4Cl. The same lysis procedure was applied, for control purposes, to the unseparated population and the T cell poor population before culture. A pure preparation of levamisole (Janssen Pharmaceutica, Belgium) was dissolved in medium RPMI 1640 with HEPES and passed through a Millipore filter to sterilize it. The final concentrations of levamisole had a range from 1 to 10^{-8} mg/100 ml. The cultures were done in plastic tubes (16 × 75 mm) containing 200,000 cells in one ml of RPMI 1640 with HEPES, L-glutamine, penicillin, streptomycin and 10% heat inactivated fetal calf serum. In some cultures, one microgramme of a PHA-P was contained in this ml of culture. To each culture was added 0·1 ml of various concentrations of levamisole. The cells were cultured, at 37°C for 3 days. Six hours before harvesting, 0·5 μCi of ^{3}H-thymidine (specific activity: 20 mCi/mmole) were added to each culture. The cells were collected on glass fiber filters (mash filters: Dynatech Lab.), washed in saline and trichloreactic acid. The filter was ethanol dried and transferred in a vial to which scintillation fluid was added. Counting was performed using a beta counter and the results were expressed in counts/min.

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Table 1. *Effect of levamisole on PHA response**

	Levamisole concentration (mg/100 ml)								No PHA and no levamisole†
	0	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Unseparated population	64·657 ± 904	66·241 ± 1·178	76·424‡ ± 773	72·341 ± 1·383	70·421 ± 1·506	68·320 ± 821	62·031 ± 1·172	61·342 ± 714	1·625 ± 67
T cell rich population	48·324 ± 730	46·920 ± 659	57·032‡ ± 510	51·910 ± 552	52·542 ± 681	49·034 ± 608	46·491 ± 495	47·031 ± 612	341 ± 14
T cell poor population	7·174 ± 206	4·324 ± 479	6·834 ± 226	5·921 ± 208	6·834 ± 231	6·901 ± 182	8·362 ± 159	7·930 ± 202	1·906 ± 252

*The results are expressed in counts/min ± S.E.M. (6 experiments).
†The cells were cultured without PHA and levamisole.
‡These results are significantly different ($P < 0·05$) from the cultures done in the absence of levamisole (first column).

RESULTS

The results are reported as the mean of the six experiments ± standard error of the mean for each investigated population (Table 1). It can be seen that a significant increase of PHA response was noted only for 10^{-3} mg/100 ml in the unseparated population and in the T cell rich population. The statistical analyses has been done using the Student's *t*-test for paired data. The level of significance is *P* less than 0·05. Statistical analysis was also done to compare the triplicate control cultures (no levamisole, PHA one microgram) to each other culture. In the unseparated population, levamisole at 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} mg/100 ml increased significantly the response to PHA in only one instance out of six (not the same experiment), in 2 experiments out of 6 at 10^{-2} mg/100 ml, in 4 experiments out of 6 at 10^{-3} mg/100 ml. Similarly, for the T cell rich population, levamisole significantly increased the PHA response 4 times out of 6 for 10^{-3} mg/100 ml, 2 times out of 6 for 10^{-4} and 10^{-7} mg/100 ml in one experiment for 10^{-5} mg/100 ml. In the T cell poor population, levamisole increased the PHA response in only one instance at 10^{-3} mg/100 ml. Levamisole, by itself, in the absence of PHA, never stimulated any of the lymphocyte populations.

DISCUSSION

These experiments, done to investigate the possible, *in vitro*, action of levamisole on human lymphocytes from normal individuals, clearly indicate that, in rather strict conditions, the drug influences the thymidine uptake in presence of PHA. Levamisole, by itself, was not mitogenic for human lymphocytes. This observation is thus different from the situation in mouse where levamisole can be slightly mitogenic [6, 7]. Similar results have recently been published by Hadden *et al.* [7]. On the other hand, levamisole will significantly increase the PHA response of human lymphocytes. This effect is however not very dramatic since the increase is only of 15–20%. Furthermore, it was obtained only at a concentration of 10^{-3} mg/100 ml (roughly 10^{-7} M). This small and narrow ranged action of levamisole on PHA response probably explains the difficulties of many investigators to demonstrate this phenomenon although recently, similar data have been reported [7].

This action is also detected on isolated blood T cell rich populations. The increase is also in the range of 20%. In contrast, levamisole did not increase the PHA responsiveness of T cell poor populations. These results are thus similar to the mice system where pure T cell populations increased by 40% (at 10^{-7} M) the response to PHA [7]. The fact that, in the mouse system, the T cells were isolated from the thymus rather than the blood, the purity of various preparations and T cell heterogeneity may explain these slight discrepancies. These studies have thus shown that levamisole can act on proliferative human T cells. It remains, however, unclear whether the, *in vitro*, experiments can explain the, *in vivo*, observed effects. Indeed the, *in vitro*, range of activity is very small and restricted to higher concentrations than in the, *in vivo*, situation. It is possible that the activity of levamisole, in low concentrations, is party due to its metabolites.

Finally, these data suggest that either levamisole acts preferentially on a stimulated (proliferative) T cell, suggesting some type of antigenic stimulation as a prerequisite for levamisole activity or levamisole enhances the immune response as reflected in the response to mitogens.

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A Preliminary Clinical Study of the Use of Mycophenolic Acid as a Radiosensitizer

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Abstract—*Animal experiments have suggested that the drug Mycophenolic Acid may be a radiosensitizer.*

This brief communication describes a clinical trial of the drug in patients with two or more similar skin metastases or with bilateral lung metastases. The patients acted as their own controls, receiving two types of treatment on separate occasions using either Radiotherapy alone or Radiotherapy with Mycophenolic Acid.

The study failed to show synergism between radiotherapy and the drug in metastatic tumours of the breast, stomach, caecum and testes.

INTRODUCTION

BOTH animal experiments [1] and clinical experience [2] at the Lilly Research Centre have suggested that the drug Mycophenolic acid may be a radiosensitizer, i.e. a drug which improves the ratio of the damage of tumour cells to the damage of normal cells by ionising radiation.

This preliminary clinical study of patients with metastatic tumours of the breast, stomach, caecum and testis did not confirm these findings.

MATERIAL AND METHODS

Selection of Patients

Patients were selected because they had either two or more similar skin metastases or bilateral lung metastases from a histologically proven primary tumour. Thus, patients acted as their own controls. They were excluded from the trial if they were clinically unfit for radiotherapy or if the lesions under study were in a previously irradiated zone.

Treatment

Each patient received two types of treatment on separate occasions to similar skin or lung metastases, using either radiotherapy alone or radiotherapy with Mycophenolic acid.

Metastatic skin nodules. Pairs of similar lesions were irradiated to the same dose with superficial X-ray therapy (70 kV, 9 mA, H.V.L. 1·5 mm Al.). A dose of 800, 900 or 1000 R in a single treatment was used for pairs of small lesions up to 2 cm dia. Larger metastases were given 1200, 1300 or 1400 R in 2 treatments over 4–7 days.

During the treatment of one of each pair of lesions oral Mycophenolic acid was given. The drug was started 2 full days before treatment and continued throughout the day or course of treatment. Daily doses ranging from 800 to 2400 mg were given for periods of 3–9 days.

Bilateral lung metastases. Each lung was irradiated with a separate course of Cobalt 60 teletherapy (1·2 MeV, H.V.L. 10·4 mm Pb), one with and one without concurrent intravenous sodium mycophenolate. Two patients were treated. One patient had metastases from a radiosensitive seminoma of testis. He received a maximum tumour dose of 1000 R in 6 treatments over 9 days. The other had metastases from a radioresistant adenocarcinoma of the caecum. She received a maximum tumour dose of 2000 R in 7 treatments over 18 days.

Both patients started intravenous sodium mycophenolate 4 g in 5% Dextrose given over a period of 4 hr each day 2 days before treatment of their right lung. The patient with the seminoma continued this dose daily throughout the treatment course but the other patient, after the first two days, was only prepared to accept an intravenous drip on the day of treatment. Both patients completed the intravenous infusion of sodium mycophenolate

approximately 1 hr before each radiotherapy treatment.

PATIENT RECORDS

A detailed assessment of the present condition, including a description of the lesions to be treated was made before each treatment course. Haematological and biochemical findings were recorded together with diagrams, photographs and X-rays as appropriate.

On completion of treatment and at 1 week, 1 month, 3 months and 6 months after treatment, changes in the treated lesions and in the adjacent irradiated normal tissues, e.g., skin, subcutaneous tissue and lung were observed, compared and recorded with photographs and X-rays if appropriate. A full blood count and biochemical examination were carried out and changes noted.

RESULTS

Skin metastases. Eighteen pairs of skin metastases were treated in 13 patients. Except for one patient who has a carcinoma of the stomach, the rest (17 pairs) had breast carcinoma. The tumour response with and without Mycophenolic acid was similar. The radiation effect on the skin ranged from a mild erythema to a dusky erythema and was similar in both treatment groups. No patient developed any late subcutaneous fibrosis.

Bilateral lung metastases. The first patient treated was a co-operative male journalist aged 53 with metastases from a seminoma of the testis in his intra-abdominal lymph nodes and in both lungs. His left lung was irradiated first to 1000 R. On completion of treatment there had already been a dramatic improvement. His right lung was irradiated similarly but with daily intravenous sodium mycophenolate, again with a dramatic and similar improvement. At exactly one week and approximately 5 weeks after the completion of each course, regression was similar on both sides. He died soon after.

The second patient was a frail elderly lady with bilateral lung metastases, worse on the left side, from an adenocarcinoma of the caecum which had been successfully excised 2½ years previously. Adenocarcinoma cells were present in her sputum. The left lung was irradiated first to a maximum tumour dose of 2000 R with little effect. Similar irradiation was delivered to the right lung with sodium mycophenolate. She received 4 g daily for two days before treatment and then 4 g on the 7 days she was irradiated. The left lung showed some improvement on X-ray at 1 and 2 months after the completion of treatment but then deteriorated. In the right lung which had been irradiated with sodium mycophenolate, the metastases were larger at each time interval.

Neither patient developed a radiation pneumonitis or any other evidence of increased radiosensitivity of the normal tissues.

DRUG TOXICITY

Neither of the patients given sodium mycophenolate experienced toxic symptoms during or after administration.

Oral Mycophenolic acid caused nausea in 8 of 13 patients. The incidence and severity of nausea was related to the daily dose. The experience of this trial suggested that a daily dose of 1200 mg is the maximum which patients will tolerate. Neither haematological nor iochemical changes were produced in any patient.

DISCUSSION

Perhaps with the exception of the development of Neutron therapy, radiotherapy alone is unlikely to offer any major contribution to cancer therapy in the forseeable future. The most likely major advances may be in the field of radiosensitizing drugs.

A radiosensitizer is a chemical which increases the cell-killing effect of a given dose of radiotherapy, but to be of any value in radiotherapy a radiosensitizer must improve the therapeutic ratio (i.e., the ratio of the damage of tumour cells to the damage of normal cells)—otherwise the same effect could be produced simply by increasing the dose of radiation.

There are very few true radiosensitizers and most of the drugs for which this claim has been made have acted by addative rather than by synergistic effect with radiotherapy. Oxygen is the most effective radiosensitizer discovered so far and the most fruitful laboratory research at the moment appears to be in the use of electron-affinic compounds, as yet too toxic for use in man, which act in a similar way to oxygen.

Preliminary work by Sweeney and his colleagues [1] showed synergism between Mycophenolic acid and X-ray therapy in the treatment of Gardner lymphosarcoma in mice. He was not able to show synergism in all tumour type and suggested that synergism was related to high β glucuronidase activity.

This pilot study has failed to show a similar

synergism in the treatment of four human tumours, i.e., carcinoma of the breast, stomach and caecum and seminoma of the testis. It is suggested that this simple clinical experiment could be used to test other possible radiosensitizing drugs.

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A Virological Study of Interstitial Pneumoniae in Patients with Acute Lymphoid Leukemia Treated with a Combination of Methotrexate and 6-Mercapto Purine

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Abstract—*A clinical study of 45 patients and a virological survey of 34 patients with acute lymphoblastic leukemia have been undertaken to detail the characteristics of occurrence of interstitial pneumoniae (IP) under maintenance chemotherapy. Our study confirms the link between IP and treatment with MTX but does not support the hypothesis of an allergic reaction to the drug. The virological study has failed to detect one type of viral agent associated with IP but have shown important modifications of humoral immunity against viruses.*

INTRODUCTION

INFECTION represents the major cause of death in patients with acute leukemia [1, 2] and the lower respiratory tract is the most common localization of infectious agents [3].

In recent years, the association between antileukemic drugs especially Methotrexate (MTX) [4] and interstitial pneumoniae (IP) has emerged as a new clinical entity. The pathogenesis of this pulmonary complication is still a subject of controversy and various causes, such as: allergy to MTX [5], infections by cytomegalovirus or pheumocystis carinii have been evoked. This study was undertaken to detail the clinical, immunological and microbiologic characteristics of IP.

Preliminary results of this study have been published [6]. We report here the results of clinical studies on 45 patients and of the virologic survey of 34 patients with acute lymphoid leukemia.

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§We acknowledge the technical assistance of Y. Augery, M. H. Lourenco-Batista and A. M. de Souto-Almeida, and we thank A. Beaudiment for expert secretarial assistance.

PATIENTS AND MÊTHODS

This study has been done in patients with acute lymphoblastic leukemia who were admitted in the department of Hematology of Institute Gustave-Roussy from November 1971 to January 1974.

The diagnosis of interstitial pneumoniae was made on chest roentgenograms which showed typically bilateral perihilar and rapidly extensive pulmonary infiltration. Usually, these radiological findings were associated with fever, dyspnea but few if any physical signs. In two cases, this interstitial pneumoniae caused the death of the patient. In all other cases, a complete regression was obtained after stopping the administration of MTX and administration of prednisone. Strikingly, we have never observed in our patients the recurrences of IP, even under prolonged periods of treatments with MTX-6MP.

The techniques used for the virological study have been previously described [6]. In all patients, a systematic study during and after the period of treatment with MTX and 6-mercapto purine (GMP) was undertaken.

Surveillance cultures were regularly obtained from throat and urine samples. A serological study of antibodies against 23 viruses was done

at weekly intervals. Statistical analysis were performed with the Chi square test.

RESULTS

Clinical study: population characteristics

During a period of 14 months from November 1971 to December 1972, 18 IP occurred among 45 patients (40%). The sex ratios of male and female patients were 1·7 for IP^- and 0·5 for IP^+, (difference not statistically significant); 17 IP^- and 14 IP^+ patients were in the first perceptible phase of the disease. Other patients had previously relapsed one or more times. The median age was 9 years for IP^- and 7 years for IP^+ patients. Under systemic MTX-6PM associated with intrathecal injections of MTX, 2 IP occurred at the end of the MTX-Prednisone phase and 1 during Vincristine-MTX.

The absolute and relative level of eosinophils in the blood of patients was studied during and after the phase of treatment with MTX-6MP.

As shown in Table 1, a significant increase of eosinophils/mm^3 was observed in patients treated with MTX-6MP, but there was no difference between patients with IP (IP^+) and patients without IP (IP^-).

In order to detect a direct toxicity of MTX, biologic tests of liver functions and the effect of intrathecal injections of MTX on proteins and cells in the cerebrospinal fluid (CSF) were followed. Liver function tests were usually normal. A cytological reaction with raised levels of proteins in CSF was observed in both IP^+ and IP^- (results not shown).

Virological study

The virological study has been completed in 34 patients. In 4 out of 11 patients IP^-, 5 viruses have been isolated: 3 reovirus type 1, 1 parainfluenza 3 and 1 herpes type 1. In 3 out of 23 patients IP^-, 3 viruses have been identified: 2 adenovirus and 1 cytomegalovirus. The difference of frequency of virus isolation between IP^+ and IP^- patients is not significant.

The results of the serological study of these patients are shown in Fig. 1. The main findings can be summarized as follows: (1) many serological modifications (rise in titres or seroconversion) have been observed in patients treated by 6MP-MTX; (2) there are simultaneous rises of antibodies against unrelated types of virus; (3) the same serological variations have been found in IP^+ and IP^- patients; (4) all patients from whom a virus could be isolated did not develop detectable amounts of antibodies against this virus, even after prolonged periods of observation; (5) simultaneous seroconversions against rubella virus have been noted in 5 patients who did not develop subsequently interstitial pneumonia.

Table 1. *Blood eosinophily and interstitial pheumoniae (IP) in all patients treated with 6MP-MTX*

Patients without interstitial pneumonia (27 patients)			
Not treated with 6MP-MTX	$67·3 \pm 25·2/mm^3$	or	$1·29\% \pm 0·5$
Under 6MP-MTX	$28·5 \pm 70$	or	$5·18\% \pm 1·4$
	statistically significant $P > 0·01$		
Patients with interstitial pneumonia (18 patients)			
Treated with 6MP-MTX before the occurrence of IP	$376·7 \pm 196$	or	$5·76\% \pm 2·8$
During the IP	$388·7 \pm 186$	or	$6·47\% \pm 3·6$

The difference of eosinophily between IP^- and IP^+ patients treated with 6MP is not significative.

DISCUSSION AND CONCLUSION

The clinical and virological results reported here bring some interesting conclusions concerning the physiopathology of IP in leukemic patients treated with MTX. The allergic reaction suggested by Clarysse *et al.* is very unlikely. Eosinophily is observed in patients treated with MTX, but there are no differences between IP^+ and IP^- patients. In addition, after regression of the IP, MTX can be readministered with safety to the same patient. We have not found signs of a direct cytotoxicity of MTX on liver and CSF, but this does not exclude a toxicity of this agent on lung parenchyme or on associated cell populations (as macrophages for example). The absence of recurrence of IP even after prolonged period of administration of MTX favour the hypothesis of an immune status to an infectious etiologic agent. The frequent isolation of pneumocystis carinii in lung biopsies of patients

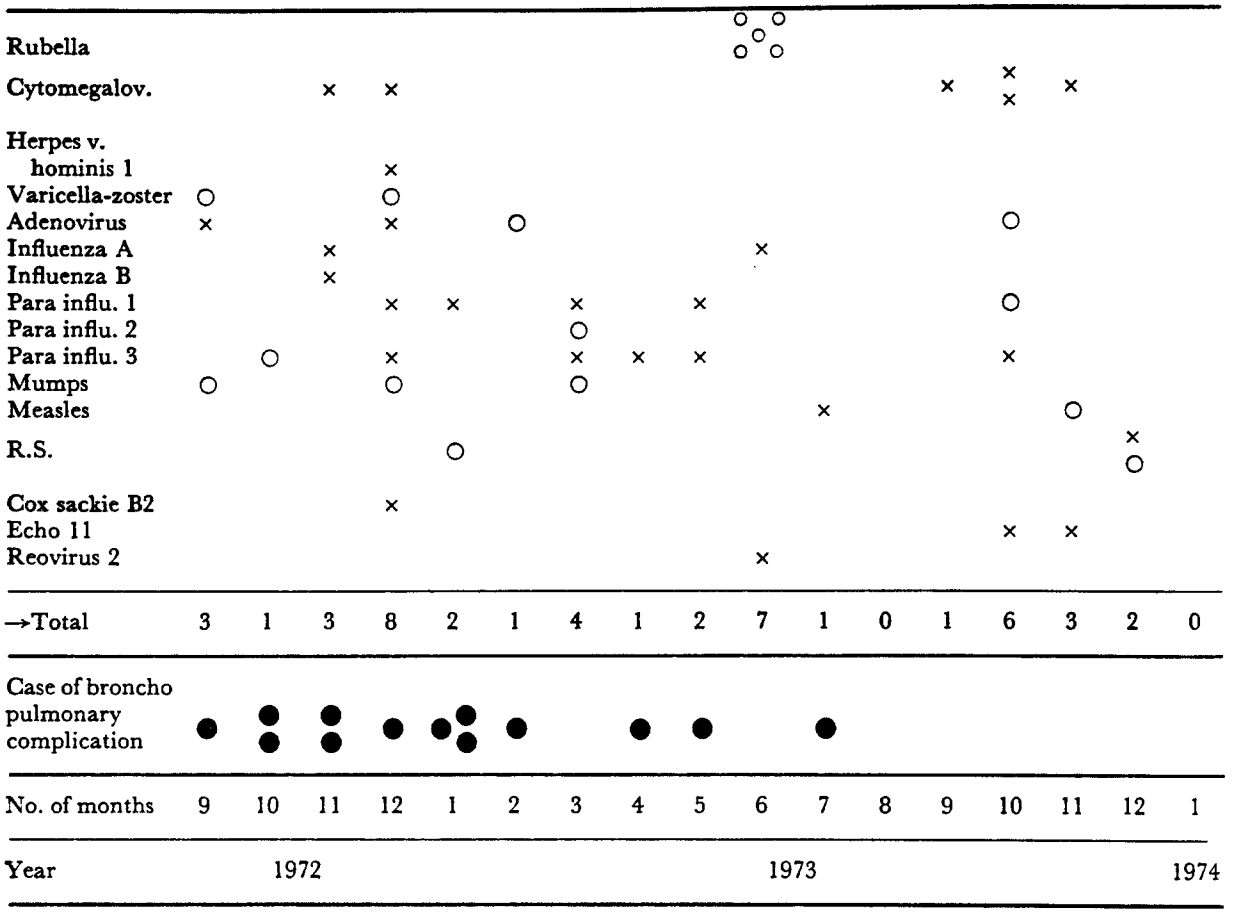

Fig. 1. *Distribution of virus antibody rises in all patients treated with* 6*MP-MTX.*

○ *Seroconversion stricto sensu.*
× *Rise in titer of antibodies already detected before treatment.*

with IP have been reported by some authors. We have not performed lung biopsies in our patients, but the regression obtained without any specific treatment of pneumocystis carinii infections argues against this etiology in our serie. The results of our virological study confirm the existence of important perturbations of humoral immunity in patients treated with immunodepressive agents like MTX and 6MP. The absence of antibodies in all 7 patients from whom a virus was isolated is striking. On the other hand, simultaneous increase of antibodies levels against unrelated types of virus (as in patients with kidney transplants) have been observed. The isolation in 3 patients of reovirus type 1 which is rarely found in humans and simultaneous seroconversions against rubella virus in patients demonstrate the frequence of horizontal transmission of viruses in these patients. However, these contaminations did not correlate with clinical and radiologic signs of IP.

In conclusion, this virological study of all patients treated with MTX demonstrates the existence of important modifications of humoral immunity against viruses. It is therefore probable that it exists similar modifications of immunity against other infectious agents such as pneumocystis carinii. The possible role of cellular immunity and of a direct or indirect toxic effect of MTX on lung deserves further investigations.

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Blood and Bone Marrow Response Following Total Body Irradiation in Patients with Lymphosarcomas

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Abstract—*Marrow depression and associated peripheral blood changes following fractionated T.B.I. are considerable and appear alarming. However, provided the marrow reserve is good and is not compromised by previous chemotherapy and radiation therapy, recovery occurred in all cases and appeared to be complete. Bone marrow of 3 patients with previous T.B.I. did not show recovery after the second course of T.B.I. Extreme caution is indicated when such a therapy is repeated, as this may lead to progressive marrow hypoplasia. Fractionated low dose T.B.I. could be utilized as a useful therapeutic modality in the management of disseminated lymphosarcoma provided the marrow reserve is good.*

INTRODUCTION

DURING recent years whole body radiation has attracted considerable attention. This form of therapy has been used particularly in the management of non-Hodgkin lymphoma [1, 2] and chronic lymphatic leukaemias [3].

In practical localized radiation therapy serious damage of blood seldom occurs. The dangers, however, are increasing as radiological advances introduce larger and larger total dose. With some techniques, such as internal radioactive isotope administration or total body irradiation (T.B.I.), the limiting factor in dosage has already shifted from cutaneous tolerance to haemopoietic tolerance.

Within the last two years 19 patients with lymphosarcomas have been treated with T.B.I. An attempt was made to study the peripheral blood and bone marrow changes in patients so treated. The result of this study is presented below.

MATERIAL AND METHODS

Nineteen patients with lymphosarcoma have been treated by T.B.I. There were 2 stage III and 17 stage IV patients. Nine of these patients had previous chemotherapy and some form of radiation therapy prior to T.B.I. and had some degree of marrow depression. Ten patients, who had no prior therapy, had normal bone marrow activity. The T.B.I. induction course consisted of 10 rads midplane 3 times weekly to a total of 100-300 rads on 6 MeV linear accelerator. The patients were positioned in an attitude of "universal flexion" over a chair and treated alternatively from left and right side. Midplane dose was checked by midrectal dose measurement with lithium fluoride dosimeter, which showed a high degree of correlation to physical dose measurement.

Blood counts were done before each treatment and treatment was stopped if the thrombocytes fell below 50,000 and leucocytes below 2000/cm. Bone marrow study was done before starting the treatment and repeated, if possible, after 100 rads midplane dose was given, and then 2 and 6 weeks after the end of the treatment. In some cases marrow study was done on the last day of therapy. Pre-irradiation of bone marrow was used as a control for further comparison.

All patients received a single induction course of T.B.I. except 3, who received a second course of T.B.I. after an interval of 14 months. Most patients received a radiation dose varying between 180–220 rads.

RESULTS

The effect of T.B.I. on bone marrow and blood was observed. The general sequence of events were as follows.

Changes in the peripheral blood following the first course of T.B.I.

The change began to appear after one week of therapy.

Thrombocytopenia was first to develop and this was very striking. The fall in the total count was gradual in most patients. In a few, however, it was sudden and sharp.

Lymphocytopenia (in patients, who had previously no peripheral blood involvement) accompanied thrombocytopenia.

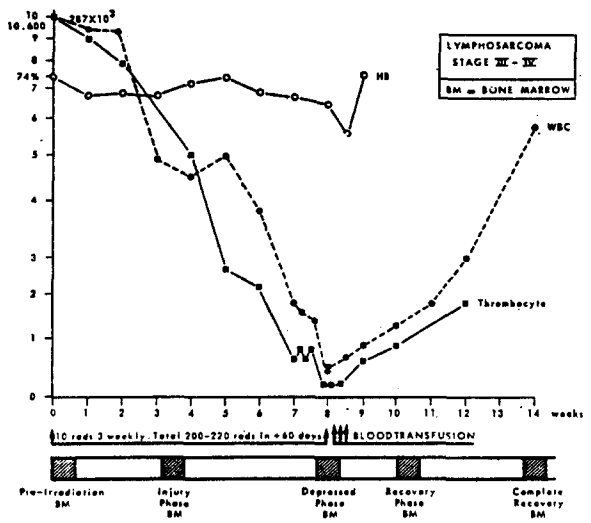

Fig. 1. Total body irradiation. Effect on peripheral blood count and bone marrow.

Granulocytopenia developed more slowly. In most cases the drop in the count was noted in the second week of therapy.

Erythrocytes count was very little affected. Total count remained almost to the pre-irradiation level throughout.

Haemoglobin was also very little affected. The decline in haemoglobin level was minimal except in those who developped auto-immune haemolysis. The haematological depression continued in a progressive manner for at least 2 weeks after the end of T.B.I. Granulocytes recovered first. Thrombocytes and lymphocytes recovered slowly, but completely. The total leucocyte count gradually increased to pre-irradiation level in 3–4 weeks except in one patient, who died soon after T.B.I. due to severe infection and bleeding. By the end of 4 weeks all the elements of peripheral blood had returned to normal in all patients except 3 who had two courses of T.B.I. In these 3 patients the count has remained well below the pre-irradiation level. They have now developed progressive anaemia and thrombopenia. Patients with previous chemotherapy and localized radiation therapy also showed delayed recovery of their peripheral counts.

Marrow changes following the first course of T.B.I.

After receiving 100 rads midplane the marrow was found to be less cellular as compared to the pre-irradiation examination (comparison of bone marrow slides is difficult as no two slides are alike nor are the marrow samples taken from two different sites and this must be borne in mind while making conclusion). Immature blood-forming cells appeared to be reduced as there was clear reduction in number of more matured cells. The formed and matured elements had a normal aspect. No change was seen in the pathological lymphocytes. Bone marrow after receiving 220 rads midplane showed further reduction of cellularity. Developed megakaryocytes were also much less in number and in one case megakaryocytes showed evidence of cell damage.

Again no change was seen in the pathological lymphocytes.

Bone marrow, 2 weeks after the end of T.B.I., showed regenerative changes; the marrow was much more cellular and large numbers of premature haemopoietic cells were seen. There was also an increase in the number of more formed elements of all the haemopoietic series.

Bone marrow, 6 weeks after the end of T.B.I., was found to be highly cellular; the cellularity appeared more marked as compared to pre-irradiation level. Formed elements of haemopoietic series were in abundance. In all aspects the marrow appeared normal and had recovered completely. Pathological lymphocytes again did not show any change. In 3 patients with two courses of T.B.I., the marrow recovery was not complete. The marrow remained less cellular and hypoplastic and hypoplasia is now progressive with again many pathological lymphocytes.

DISCUSSION

T.B.I. produces early dramatic changes in the peripheral blood, especially in thrombocytes. These changes are chiefly a result of damage to the haemopoietic immature cells and are not a direct effect on the circulating cells [4–5]. Radiation damage interrupts the balance by reducing the supply of blood cells in the circulation leading to pancytopenia. These peripheral changes are in turn dependent on the severity of the haemopoietic damage. With sublethal dose given in small fractions over several weeks, similar changes, but of a lesser degree, were also observed in this study. Bone marrow showed partial cessation of

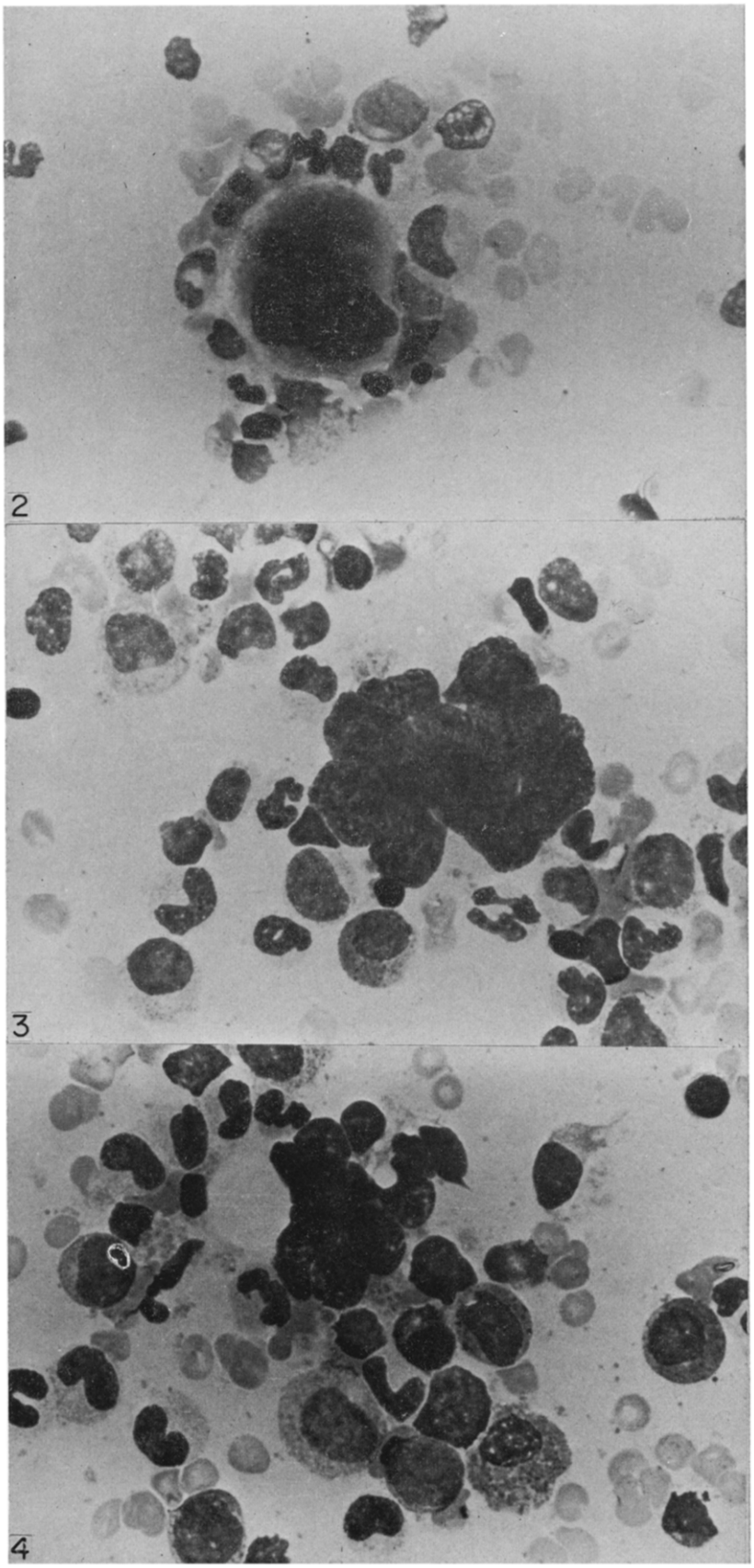

Fig. 2. *Normal megakaryocytes High magnification.*

Figs. 3 *and* 4. *Marrow following T.B.I. showing damaged megakaryocytes.*

Please note: surrounding cells are intact, so artefact is less likely.

proliferation and destruction of sensitive primitive cells. The cellularity was decreased. Matured elements were also much reduced. These changes were progressive and, by the end of therapy, having received up to 220 rads, the cellularity was reduced considerably. Pathological lymphocytes however, remained unchanged throughout.

Once the radiation was stopped, marrow recovery began and, by the end of 2 weeks, large numbers of primitive haemopoietic cells were seen in the marrow. Formed elements were also increased. Apparent complete marrow recovery was seen at the end of 6 weeks after T.B.I. Bone marrow damaged by previous T.B.I. did not show complete recovery as was seen in the 3 patients, who had a second course of T.B.I. Nine patients with previous therapy showed delayed recovery.

Changes in the peripheral blood could be related to the marrow changes. The noticeable change was observed one week after the therapy was started. Thrombocytopenia was the first to be observed. The fall in thrombocytes, normal lymphocytes and granulocytes, was progressive and continued until at least 2 weeks after the end of T.B.I. By the end of 4 weeks the count had returned to pre-irradiation level except in those patients with previous therapy and in 3 patients with a second course of T.B.I. The entire peripheral blood changes could be attributed to maturation-depletion as a result of the failure of cell renewal from more primitive sources in the marrow.

Perhaps the number of haemopoietic stem-cells (HSC) in the body in normal condition remains relatively constant despite continuing demands for differentiation into cells of myeloid, erythroid and megakaryocytic series [6]. Recovery of HSC following irradiation indicates the existence of a sensitive haemostatic mechanism for its repopulation.

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Combination of Radiotherapy and Immunotherapy in the Treatment of Lewis's Tumour

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Abstract—*Three groups of 6–8 week old male C57 Bl/6 x DBA2 F1 mice with transplanted Lewis tumour were irradiated by a single dose of Cobalt 60 localized on the tumour in association with intraperitoneal injections of BCG. When the animals were sacrificed, tumour parameters were determined: weight, surface area, volume and lung metastasis. When BCG was applied up to and including the 4th day following the end of the irradiation, tumour growth and the number of lung metastases were significantly decreased in comparison with the control group ($P < 0.01$). In all the groups where BCG treatment was started after the 4th day post-irradiation, there was no effect of BCG when compared with radiotherapy alone. This emphasizes the importance of timing in radiotherapy and immunotherapy association.*

INTRODUCTION

SURGERY and radiotherapy are actually the primary treatment for cancer with chemotherapy and immunotherapy constituting complementary and effectual therapies. Nevertheless, the problem is that most of these therapeutics are immunodepressive. Radiotherapy can encourage the implantation of metastasis [1, 2]. The effect of immunotherapy is actually well-established [3-5] and the association of chemotherapy and immunotherapy [6] seems most favourable in a certain number of tumours. However, the problem remains as to the moment when immunotherapy can be applied relative to chemotherapy [7-9]

The task of this research has been to establish a competent association of radiotherapy and BCG in Lewis tumour by utilising variable dates at the moment of the BCG application after radiotherapy. Consequently it appeared very distinctly that only the BCG applied up to and including the 4 days following the termination of the radiotherapy is effective.

MATERIAL AND METHODS

(1) *The animals*

Six to eight week old male (C57 Bl/6 x DBA2)F1 mice were used for this experiment. The Lewis's tumour is well maintained in our Laboratory and was kindly supplied by the I.C.I.G. (Pr. Mathé). It was implanted into the animals left paw. 2×10^6 viable cells were injected in 0·2 ml of MEM by the subcutaneous route.

(2) *The irradiation*

A local irradiation was performed on the paw where the tumour was injected. Radiation was systematically applied 8 days after the tumour implantation which corresponds to the 4th day after the actual appearance of the implanted tumour, the average tumour surface area being then 120 ± 15 mm^2. The irradiation used a source of Cobalt 60 with an irradiation of 70 rads/min in air at a source-skin distance of 60 cm. The dose delivered to the paw was a unique dose of 2100 rads utilising a fixed field.

(3) *The BCG*

The BCG from the Pasteur Institute with a dose of 1 mg injected i.p. in 0·2 ml of physiological serum was employed.

(4) *The tumour*

The animals were systematically sacrificed the 14th day in the group 1, the 23rd day in the group 2 and the 30th day in the group 3. The weight, the surface area and the volume of the tumours were accordingly evaluated as well as the number of pulmonary metastasis.

(5) *The groups*

The group 1 consisted of 5 sub-groups. Sub-group A was the control sub-group where the animals with the tumour were treated by irradiation, but had not received the BCG; sub-group B received 3 injections of BCG, 2, 5 and 8 days after the tumour implantation and consequently before the irradiation; sub-group C received BCG injections the 1st, 4th and 6th days after the irradiation; sub-group D received the BCG the 4th and 6th day after irradiation; and finally the sub-group E received an injection of BCG 6 days post-irradiation.

In the group 2, the sub-group A was the control; the sub-group B received the BCG the 1st, 4th, 6th, 9th and 13th days post-irradiation; the sub-group C received the BCG injections the 4th, 6th, 9th and 13th days post-irradiation; sub-group D received the BCG injections the 6th, 9th and 13th days post-irradiation; and finally the sub-group E received the BCG injections the 9th and 13th days post-irradiation

In the group 3, the sub-group A was the control; the sub-group B received the BCG injections the 1st, 4th, 6th, 13th, 18th and 26th days after the irradiation; the sub-group C received the BCG the 4th, 6th, 13th, 18th, and 26th days post-irradiation; the sub-group D received the BCG the 6th, 13th, 18th and 26th days post-irradiation; and finally the sub-group E received the BCG injections 13, 18 and 26 days post-irradiation. For each one of these groups a non-treated control tumour was realised. The results were interpreted by statistics on a Multi-4 Intertechnique computer using the Student–Cochran test ($P < 0{\cdot}05$: statistically significant).

RESULTS

(1) *Group* 1 (*Table* 1)

The increase of the tumour surface area in the sub-groups B and C was less important in relation to the control. On the other hand, no statistical difference was noted in the sub-groups D and E. There was a diminution in the tumourous weight of the sub-groups B and C, but this was only significantly noticable in the sub-group C. This applied also to the tumourous volume. Finally, it was noted that there was a significant reduction of the number of pulmonary metastasis in the sub-groups B and C.

(2) *Group* 2 (*Table* 1)

The tumour surface was noticably reduced in the sub-groups B and C. The weight and the volume of the tumours were also reduced for sub-groups B and C and it was noted that there was an important increase in these parameters for the sub-groups D and E compared to the control. Finally, the number of pulmonary metastasis was also significantly diminished in the sub-groups B and C.

(3) *Group* 3 (*Table* 1)

The results were similar enough to those observed in the groups 1 and 2; namely a definite diminution of the surface, weight and volume of the tumour for the sub-group C. The same observation applied to the pulmonary metastasis.

DISCUSSION

The study of these results delineates a significant diminution of the tumourous growth in

Table 1. *This table shows the results for the* 3 *groups of tumour weight and pulmonary metastasis. The data shows the interest of BCG administration up to and including the* 4*th day post-irradiation. A′ is a control sub-group without any treatment*

	Tumor weight*			Number of pulmonary metastasis†		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
Sub-groups						
A	3·32 ± 0·84	5·3 ± 1	6·8 ± 0·75	15·3 ± 0·9	85 ± 2	85 ± 1·5
A′	6·13 ± 0·86	8·92 ± 1·06	10·5 ± 2·4	35 ± 0·9	82 ± 1	78 ± 1·4
B	2·77 ± 0·50‡	3·4 ± 0·7§	5·9 ± 0·8‡	12·5 ± 1·03	55 ± 1·5§	75 ± 1·3
C	2·05 ± 0·7§	3 ± 0·5§	5·1 ± 0·7§	5 ± 0·7‖	37 ± 1·5‖	45 ± 1·4§
D	2·38 ± 1·05‡	7·1 ± 0·8‡	7 ± 0·9‡	17·5 ± 1·1	73·1 ± 0·8	75 ± 1·3
E	3·42 ± 1·04‡	7·4 ± 1·1‡	7·3 ± 0·9‡	17·6 ± 1·7	75 ± 0·7	85 ± 0·9

*in g (Mean ± S.E.).
†Mean ± S.E.
‡Non significant: $P > 0{\cdot}05$.
§Statistically significant: $P < 0{\cdot}05$.
‖Statistically significant: $P < 0{\cdot}01$.

the animals treated by BCG before and after irradiation. However, when the BCG is given after irradiation, the diminution of the tumourous growth is only observed when this treatment is started up to and including the 4th day following the end of this irradiation. Moreover the number of BCG injections had no influence either on the tumourous growth or on the number of pulmonary metastasis. It is noticed that in certain sub-groups, where the BCG was performed 4 days after the end of the irradiation, an increase was noticed in the growth of the tumour.

These results emphasize once again the importance of the timing of the BCG injection in relation to the immunodepressor treatment and the number of injections realised. This fact revealed by Currie [7] has been emphasized again recently by both Milas [8] who used corynebacterium granulosum and by Mathe [9, 10]. In this way, we must re-state that BCG applied up to and including the 4th day following the end of irradiation is essential.

These results appear to be extremely important considering that it was recently emphasized that Radiotherapy was immunodepressive [1, 11, 12] and could encourage the growth of metastasis [2]. This fact, that the association of Radiotherapy and BCG reduces the number of metastases in a rapidly evolutional system such as the Lewis's tumour, emphasizes the potential interest of the association of Radiotherapy and Immunotherapy in the measure that the timing has been perfectly defined.

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Immunostimulation by *Brucella Abortus*. Role of Surface Antigenicity of the Bacteria*

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Abstract—*Two suspensions of inactivated* B. abortus *organisms prepared from either the smooth strain B19S or from its rough mutant B19R were compared as to their ability to modulate the mouse immune response against sheep red blood cells (SRBC) antigens. The anti SRBC response was measured by counting the number of spleen cells forming hemolytic foci in agar, 4 days after immunization. Referring to this test B. abortus B19R revealed more immuno-stimulant than B19S. When SRBC and B19R were injected at close time intervals, low doses of bacterial organisms were sufficient to enhance the immune response, the optimal effect being obtained for a simultaneous administration of both materials. When SRBC were given 5 or 10 days after Brucella higher doses of organisms were required to modulate the immune response and only B19R could increase the number of antibody forming cells. These results were correlated with the difference in surface antigenicity between B19R and B19S and with the cellular proliferation induced by the two bacterial preparations.*

INTRODUCTION

KILLED *Brucella abortus* organisms, strain B19 grown in smooth phase (B19S), have been shown to increase the survival time of tumour bearing animals and to enhance the antibody production against SRBC [1, 2]. The latter immunostimulating effect could be still improved by injecting B19S in mice previously treated by an hyperimmune anti-brucella serum [3]. The passive injection of this antiserum also delayed the specific agglutinin response against Brucella antigens. The hypothesis was therefore suggested that an inverse relationship might exist between the immunopotentiating properties of Brucella and its surface antigenicity. In the present work we have tried to confirm this view by comparing the effects of B19S to those of the rough non agglutinogen mutant B19R.

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MATERIAL AND METHODS

Animals

Female (C_{57} $Bl_6 \times DBA_2$) F_1 mice, aged 4–6 weeks, obtained from CNRS Orleans were used throughout the experiments at the rate of 6 mice per group.

Brucella abortus

The strain B19 was cultivated as described previously [1] either in smooth phase (B19S) or in rough phase (B19R), then suspended in phosphate buffered saline and heat inactivated. Bacterial suspensions were injected intravenously at variable doses expressed as dry weights of organisms.

Immune responses against B. abortus *and SRBC*

The anti Brucella agglutinins were measured in the serum with Wright's method. The response against SRBC was measured 4 days after i.v. injection of $2{\cdot}5 \times 10^8$ SRBC by the direct technique of plaque forming cells (PFC) according to Jerne and Nordin.

The number of PFC was related either to the whole spleen or to 10^6 spleen cells. The

final results were expressed as adjuvant indexes. The adjuvant indexes were calculated either from the number of PFC per spleen (AI/spleen) or from the number of PFC per 10^6 spleen cells (AI/10^6C) and in both cases they represented the ratio of the treated to control values. Student's *t*-test was used to compare the numbers of PFC per spleen or per 10^6 spleen cells.

RESULTS

Different experimental situations could be distinguished according to the relative times of injection of *B. abortus* and SRBC.

When *B. abortus* and SRBC were given together B19R was more efficient than B19S in increasing the number of PFC per spleen or per 10^6 cells (Table 1). Increasing the doses of bacteria did not provoke a proportional rise in adjuvant index. Relatively close effects were obtained with doses ranging from 30 to 1000 μg. Table 2 shows that B19R had to be injected mixed with SRBC to be fully efficient. The adjuvant index decreased when the two substances were given separately at 0·5–2 days intervals.

Another interesting experimental situation resulted from the injection of SRBC 5 days after *B. abortus*. In this case the anti SRBC response was increased but only by the use of B19R and only with elevated doses (Table 3). The injection of SRBC ten days after *B. abortus* led to a diminution of immune response, and this was particularly clearcut when B19S was used and when high doses were given.

The i.v. injection of B19S provokes important cellular modifications characterized by spleen and liver enlargement, accelerated renewal of non lymphoid bone marrow derived cells and enrichment of the body in hemopoietic stem cells [1, 4]. Table 4 shows that B19R produced a weaker splenic hypertrophy than than B19S even with doses twice as important. Moreover only B19S gave rise to detectable amounts of anti Brucella agglutinins.

Table 1. *Simultaneous injection of* B. abortus *and SRBC. Influence of the dose of B19S or B19R*

	AI/spleen			AI/10^6C		
Dose (μg)	B19S		B19R	B19S		B19R
31·25	1·45 (N.S.)*	(S.)†	2·35 (S.)*	1·00 (N.S.)*	(S.)†	1·95 (S.)*
62·5	1·65 (S.)	(S.)	2·60 (S.)	1·37 (S.)	(S.)	2·08 (S.)
125	1·13 (N.S.)	(S.)	2·61 (S.)	0·88 (N.S.)	(S.)	1·36 (S.)
250	1·41 (N.S.)	(S.)	3·48 (S.)	1·33 (N.S.)	(S.)	2·75 (S.)
500	1·09 (N.S.)	(S.)	2·33 (S.)	0·80 (N.S.)	(S.)	2·00 (S.)
1000	1·65 (S.)	(S.)	4·12 (S.)	0·92 (N.S.)	(S.)	2·49 (S.)

*Differences in the number of PFC between treated and control mice significant (S.) or non-significant (N.S.).

†Differences in the number of PFC between B19S and B19R treated mice significant (S.) or not (N.S.).

DISCUSSION

The PFC response against SRBC measured 4 days after immunization was increased either by injecting *B. abortus* B19R in mixture with SRBC or by injecting it 5 days before. The agglutinogen preparation B19S did not provoke comparable effects. This confirms the hypothesis of an inverse relationship between the expression of the surface antigens of the bacteria and the ability to potentiate this type of immune

Table 2. *Short time intervals injection of* B. abortus *B19R and SRBC*

	Time of injection of B19R in days*									
	—2		—1		—0·5		0		+1	
Dose of B19R	AI/spleen	AI/10^6C	AI/spleen	AI/10^6C	AI/spleen	AI/10^6C	AI/spleen	AI/10^6C	AI/spleen	AI/10^6C
50 μg	1·32 (N.S.)‡	1·12 (N.S.)	1·66 (S.)†	1·52 (S.)	1·60 (S.)	1·43 (N.S.)	3·09 (S.)	3·22 (S.)	1·24 (N.S.)	0·83 (N.S.)
1000 μg	2·22 (S.)	1·35 (N.S.)	1·40 (N.S.)	0·92 (N.S.)	—	nd —	3·85 (S.)	2·70 (S.)	1·27 (N.S.)	0·73 (N.S.)

*Day 0 was the time of injection of SRBC.

†Injection of B19R and SRBC in mixture.

‡Differences in the number of PFC between control and Brucella treated mice significant (S.) or non significant (N.S.).

Table 3. Injection of SRBC 5 or 10 days after B19R

Dose of B19S or B19R		5 days		10 days	
		AI/spleen	AI/10^6C	AI/spleen	AI/10^6C
50 μg	B19S	0·51 (S.)*	0·38 (S.)	0·89 (N.S.)	0·83 (N.S.)
	B19R	1·04 (N.S.)	0·99 (N.S.)	1·31 (N.S.)	1·34 (N.S.)
500 μg	B19S	0·94 (N.S.)	0·34 (S.)	0·58 (S.)	0·38 (S.)
	B19R	1·12 (N.S.)	0·82 (N.S.)	1·34 (N.S.)	0·94 (N.S.)
1000 μg	B19S	1·20 (N.S.)	0·42 (S.)	0·20 (S.)	0·05 (S.)
	B19R	3·66 (S.)	1·74 (S.)	0·38 (S.)	0·14 (S.)

*Differences in the number of PFC between Brucella treated and corresponding controls significant (S.) or not (N.S.).

Table 4. Spleen enlargement and specific agglutinin response after B. abortus *injection*

		Days after injection				
		4	7	10	14	20
Spleen weight (mg)*	500 μg B19R	206	293	206	190	120
	1000 μg B19R	203	269	353	348	159
	500 μg B19S	195	407	563	502	350
Agglutinin (mean titer)†	500 μg B19S	—	1/80th	1/80th	1/160th	1/320th

*Spleen weight of controls: 73 mg.
†No detectable agglutinin in B19R treated animals.

response. The two experimental situations in which B19R was able to increase the anti SRBC response differed greatly from one another. In the first one, corresponding to an adjuvant effect, B19R did not need to be injected at high doses, but had to be injected mixed with the antigen. In the second case an immunostimulant effect was obtained by giving SRBC 5 days after Brucella, but this time, high doses of B19R were required. Such large amounts of B19R provoked an intense cellular proliferation which could play an important part in the mechanism of immunostimulation. These cellular modifications however, do not seem sufficient to explain the results noted since B19S which induced a greater spleen enlargement than B19R did not cause a comparable increase in anti SRBC response.

The immunostimulant effect might finally result at least from two factors, one depending on the cellular proliferation and the other linked to the non expression of *B. abortus* self antigens.

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Cellular Immunity to the Mammary Tumour Virus in Mice Bearing Primary Mammary Tumours*

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Abstract—*The development of cellular immunity directed against the murine mammary tumour virus (MTV) during the growth of primary mammary tumours has been studied in BALB/c, BALB/cfC3H and GR mice, by measuring leukocyte stimulation and leukocyte adherence inhibition in the presence of purified MTV. Leukocytes react against group-specific antigens of different MTV strains in these assays. Purified Rauscher murine leukaemia virus was used as a control.*

In both assays, MTV-specific reactivity peaked at a tumour weight of about one gram; afterwards, it almost completely disappeared. It increased again to about half of the original level when the tumour reached a weight of about three grams. Peak blastogenesis of lymph node cells was observed at a tumour weight somewhat higher than when peak blastogenesis of spleen cells occurred.

The possible causes of the general decrease and oscillation in the cellular immune response during tumour growth are discussed.

INTRODUCTION

THE FAILURE to evoke cross-reactive transplantation immunity to mammary tumours in mice which are neonatally infected with the mammary tumour virus (MTV) has been regarded as indicative for true tolerance to the virus [1–7]. It has been reported, however, that neonatally infected C3H mice produce antiviral antibodies on immunization with MTV [8–10] and the tumour-bearing mice would contain precipitating antibodies to MTV in their serum [9–11].

Utilizing the colony inhibition test, Heppner [12, 13] and Heppner and Pierce [14] found that tumour-bearing mice contain lymphocytes that can arrest the growth of mammary tumour cells but they found no indication that the reactivity was directed against viral antigens. Blair *et al.* [15] claimed to have found virus-specific cellular immunity in tumour-bearing mice by means of the microcytotoxicity (MC) test. The antiviral nature could be demonstrated by the blocking of cytotoxic activity by pretreatment of spleen cells with viral antigens [16]. Also with the macrophage inhibition test virus specific reactivity in infected mice was observed [17]. In the present study cellular immunity to purified MTV has been investigated in tumour bearing mice with two different techniques.

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MATERIAL AND METHODS

Mice

The following inbred mouse strains were used: GR, carrying MTV-P; BALB/cfC3H carrying MTV-S and a BALB/c subline in which the endogenous MTV-O had been passaged [18]. Tumours were induced in these mice by forced breeding. Mice were killed by cervical dislocation; tumours, lymph nodes and the spleen were then taken aseptically. Mice which were immunized intraperitoneally with 10^8 tumour cells suspended in complete Freund's adjuvant and killed 5 days later were used as positive control animals. Male mice not older than 10 weeks served as normal controls.

Virus

The standard strain of mouse mammary tumour virus (MTV-S) was isolated from

BALB/cfC3H mammary tumours. Fifty grams of tumour tissue were homogenized in 500 ml phosphate buffered saline (PBS) in a Sorval omnimixer for 50 sec at 14,000 rev/min. The homogenate was centrifuged for 15 min at 12,000 × *g*. To the supernatant was added 1% M ethylene-diamine tetra-acetic acid (EDTA). This mixture was spun for 15 min at 12,000 × *g*. The supernatant was ultra-centrifuged for 45 min at 75,000 × *g* (Beckman 35 N rotor; 35,000 rev/min). The pellet was then resuspended in 90 ml 1·5 mM Tris–HCl buffer (pH 7·2) and spun for 15 min at 12,000 × *g*.

The resulting supernatant was centrifuged on a discontinuous gradient of 4 ml 50% sucrose (w/w), 12 ml 35% sucrose (w/w) and 8 ml 20% sucrose (w/w) in 1·5 mM Tris–HCl for 180 min at 170,000 × *g* (Beckman SW 27·1 rotor, 27,000 rev/min). Material collected from the interphase between the 50% and 35% sucrose layers was diluted with 1·5 mM Tris–HCl buffer and centrifuged twice on a linear sucrose buffer (20–50% w/w in 1·5 mM Tris–HCl) for 180 min at 17,000 × *g* in a SW 27·1 rotor. The bands at densities 1·16 to 1·18 g/ml were collected and centrifuged for 40 min at 150,000 × *g* (Beckman 50 Ti rotor, 40,000 rev/min). The pellets were resuspended in 0·025 M sucrose in 1·5 mM Tris–HCl buffer. The purified virus was stored in liquid nitrogen. All isolation steps were carried out at 4°C. Rauscher leukaemia virus (RLV) was isolated from leukemic spleens of BALB/c mice in the same manner. Virus content was estimated on the basis of the amount of protein, as measured by the Folin method. Before use as antigen, the virus was disrupted by repeated freezing and thawing.

Leukocyte adherence inhibition (LAI) test

The technique employed is a modification of the method of Halliday and Miller [19]. Killed mice were injected i.p. by means of a Pasteur pipette with 8 ml of ice-cold medium (RPMI 1640 with 100 i.u. penicillin and 100 μg streptomycin per ml) to which Heparin (5 i.u./ml) was added. The peritoneal fluid was withdrawn and reinjected several times and then collected into glass tubes. The cells were washed once by centrifugation at 200 × *g* for 10 min. They were then resuspended in medium containing 7% foetal calf serum (FCS) (Flow, Irvin, Scotland). The final cell concentration was 40×10^3 per ml. Antigen was then added and 20 μl of the suspension were pipetted into the wells of Falcon microtest 3034 plates. The plates were incubated for 2 hr at 37°C in a humidified atmosphere with 5% CO_2. Thereafter, the plates were washed three times in PBS and the adherent cells were fixed, stained and counted. At least ten wells were counted for determination of the average number of cells per well. For MTV and RLV alternating wells were taken. Percent reduction was calculated according to the formula $(1 - a/b) \times 100\%$ in which a is the average number of cells in the wells incubated with MTV and b that in the wells incubated with RLV. Statistical significance was established by means of the two-tailed Student's t-test.

Leukocyte stimulation (LS) test

All tests were performed with pooled cells from 3 mice; when tumour-bearing animals were tested, the cells were derived from 3 mice with tumours of approximately the same size. Spleen and lymph nodes were teased apart by forceps in a Petri dish containing medium. Spleen cells were centrifuged twice at 26 *g* for 10 min in order to remove erythrocytes. They were then again washed two times (200 *g*, 10 min); lymph node cells were spun 4 times at 200 *g* for 10 min. After counting, the cells were resuspended ($2{\cdot}5 \times 10^6$/ml) in medium supplemented with 20% FCS. The cultures (1 ml) were incubated in plastic tubes (75 × 12 mm, Falcon) in the presence of antigen for 80 hr at 37°C in a humidified atmosphere with 5% CO_2. After this, 0·15 μCi of 2-^{14}C-thymidine was added to each culture. Twenty-four hours later, the cells were sucked onto glass fibre filters (Whatman GF/A, Maidstone, England) and incorporation of thymidine was measured in a liquid scintillation counter.

The cultures were run in triplicate. Percentage of stimulation was calculated according to the formula $a - b/b \times 100\%$, in which a = average number of counts/min in cultures incubated with MTV and b that in cultures incubated with RLV. Statistical significance was established by means of the two-tailed Student's t-test.

RESULTS

LAI-assay

The optimum concentration of MTV in this test proved to be 10^{-4} mg viral protein per ml when leukocytes were taken from positive control animals (Fig. 1). This concentration of virus was used therefore in all experiments.

When pooled cells derived from 3 normal animals were tested, sometimes a significant

difference was found between the number of adherent cells in cultures incubated with 10^{-4} mg RLV, and those to which no antigen was added. However, no appreciable differences were found between cultures incubated with RLV and MTV. This is illustrated in Table 1; the experiments were repeated five times for every mouse strain. Also, a significant difference was never found when the normal cells were incubated with 10^{-4} mg RLV as compared to the same amount of ovalbumin (for instance: $95{\cdot}6 \pm 8{\cdot}3$ vs $94{\cdot}3 \pm 7{\cdot}9$ and $59{\cdot}7 \pm 4{\cdot}8$ vs $62{\cdot}1 \pm 3{\cdot}2$). For that reason, in LAI-tests on cells from tumour bearing animals, RLV was always used as a specificity control.

However, when individual BALB/c, BALB/cfC3H and GR mice bearing primary tumours were tested, a significant difference in the number of adherent cells between the wells with MTV and RLV was found in 18 out of 42 animals tested. Table 2 gives some representative results.

During the experiments with individual mice bearing tumours it was repeatedly observed that reactivity was highest when the tumour was small (varying from 0·5 to 1·7 g); with increasing tumour weight reactivity disappeared, whereas animals bearing very large tumours (above 3 to 4 g) showed again some reactitivity. Since differences in the chemicals used may exert some influence on the reactivity measured, individual BALB/c, BALB/cfC3H and GR mice bearing primary tumours of varying size were tested with the same antigen preparation and batch of FCS. The results are shown in Fig. 2.

It was found that reactivity was stronger in BALB/cfC3H mice than in the other two strains. Whether the leukocytes were incubated with the autologous MTV strain (MTV-S) or with homologous MTV-P made no difference (Table 3).

LS test

With leukocytes from positive control mice, the optimal conditions for stimulation, as

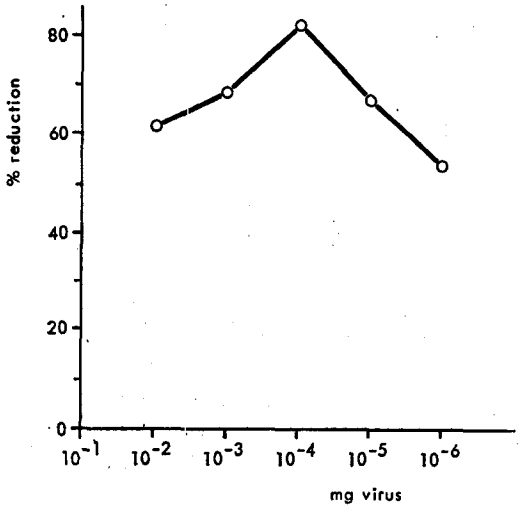

Fig. 1. Leukocyte adherence inhibition of peritoneal cells derived from positive control animals in the presence of different concentrations of MTV. This figure represents 1 out of 3 experiments.

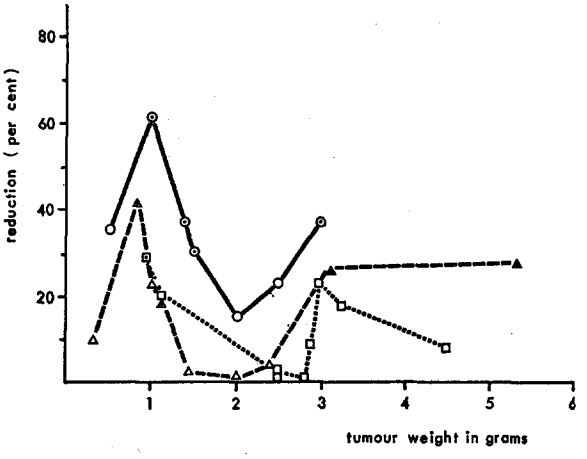

Fig. 2. MTV-specific leukocyte adherence inhibition of peritoneal cells of individual mice bearing primary tumors of variable size. All tests are performed with the same antigen preparation and batch of FCS. ○: *BALB/cfC3H*; △: *GR*; □: *BALB/c. A dot in the symbols indicate significant reduction* ($P < 0{\cdot}025$).

Table 1. Leukocyte adherence inhibition of normal mice

Mouse strain	Average No. of cells ± S.E.* adhered in medium	Average No. of cells ± S.E. adhered in the presence of RLV	Average No. of cells ± S.E. adhered in the presence of MTV	Significance of difference between RLV and medium control	Significance of difference between RLV and MTV
BALB/c	$29{\cdot}2 \pm 1{\cdot}6$	$28{\cdot}6 \pm 3{\cdot}2$	$30{\cdot}0 \pm 3{\cdot}5$	N.S.†	N.S.
BALB/cfC3H	$60{\cdot}8 \pm 4{\cdot}1$	$43{\cdot}7 \pm 3{\cdot}5$	$42{\cdot}3 \pm 2{\cdot}3$	$P < 0{\cdot}001$	N.S.
GR	$25{\cdot}6 \pm 4{\cdot}2$	$19{\cdot}6 \pm 2{\cdot}8$	$20{\cdot}3 \pm 1{\cdot}8$	N.S.	N.S.

*S.E.: standard error of ten wells.
†N.S.: not significant.

Table 2. Leukocyte adherence inhibition by MTV-S of mice bearing primary tumours

Mouse strain	MTV-strain	Tumour weight (g)	Average No. of cells ± S.E.* adhered in medium	Average No. of cells ± S.E. adhered in the presence of RLV	Average No. of cells ± S.E. adhered in the presence of MTV	% reduction (specific response)	Significance of specific response
BALB/cfC3H	MTV-S	1·0	22·9 ± 2·8	23·4 ± 3·2	9·1 ± 1·3	61	$P < 0{\cdot}001$
		3·0	38·0 ± 3·0	34·8 ± 2·7	22·0 ± 2·4	37	$P < 0{\cdot}005$
BALB/c	MTV-O	0·9	58·4 ± 2·7	45·6 ± 3·0	32·6 ± 2·1	28	$P < 0{\cdot}001$
		3·2	29·5 ± 4·0	29·0 ± 2·5	24·1 ± 1·9	17	N.S.†
GR	MTV-P	0·8	47·8 ± 3·4	43·4 ± 3·4	31·5 ± 3·2	41	$P < 0{\cdot}005$
		5·3	67·7 ± 4·0	68·5 ± 4·7	48·2 ± 1·8	29	$P < 0{\cdot}001$

*S.E. = standard error of ten walls.
†N.S. = not significant.

measured by ^{14}C-thymidine uptake, are 80 hr of incubation (Fig. 3) and 10^{-4} mg viral protein (Fig. 4).

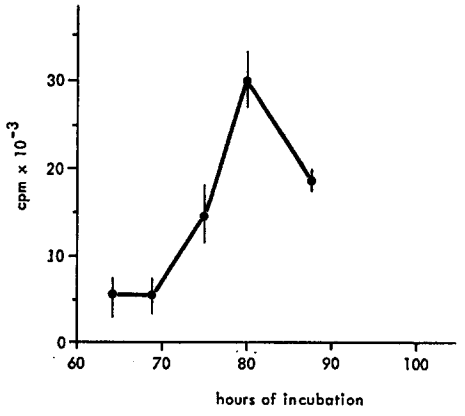

Fig. 3. Proliferation of spleen cells of positive control animals at different incubation times in the presence of MTV. This figure represents one out of 4 experiments. Percent stimulation: see text.

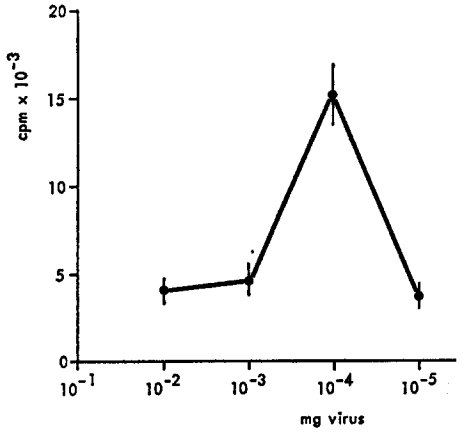

Fig. 4. Proliferation of spleen cells of positive control animals in the presence of different concentrations of MTV. This figure represents one out of 3 experiments.

From repeated experiments (3 for every mouse strain) it became clear that leukocytes from normal animals sometimes show an enhanced response with RLV as compared to the medium control experiment. This is demonstrated in Table 4. Also in this case no different values are found for RLV and MTV. The same holds true for RLV as compared to ovalbumin (for instance: 1·984 ± 489 vs 2·201 ± 312 and 2·391 ± 208 vs 2·208 ± 647). Therefore, also in this test RLV is taken as specificity control.

In contrast, in 29 out of 62 tests performed with leukocytes from tumour-bearing animals a reaction specific for MTV as compared to RLV was observed. The thymidine-uptake is 2–4 times that of the control. In Table 5

Table 3. Adherence of leukocytes from tumour-bearing GR mice in the presence of different MTV strains

Exp. No.	Average No. of cells (± S.E.)* adhered in the presence of RLV	Average No. of cells (± S.E). adhered in the presence of MTV-S	Average No. of cells (± S.E.) adhered in the presence of MTV-P
1	14·4 ± 2·4	8·0 ± 1·6	6·5 ± 0·9
2	86·8 ± 5·8	49·2 ± 3·4	45·7 ± 3·9
3	27·6 ± 1·5	25·1 ± 1·0	21·4 ± 3·0
4	42·0 ± 3·2	28·2 ± 2·1	24·1 ± 3·2

*S.E. = standard error of ten wells.

Table 4. Proliferation of spleen- and lymph-node cells of normal mice

Mouse strain	Counts/min ± S.E.* in medium control	Counts/min ± S.E. in the presence of RLV	Counts/min ± S.E. in the presence of MTV	Significance of difference between RLV and medium control	Significance of difference between RLV and MTV
BALB/c	2·010 ± 298	1·992 ± 146	1·708 ± 312	N.S.†	N.S.
GR	2·078 ± 266	3·862 ± 308	3·366 ± 352	$P < 0·025$	N.S.
BALB/cfC3H	3·619 ± 72	7·912 ± 518	7·334 ± 405	$P < 0·001$	N.S.

*S.E. = standard error of triplicate cultures.
†N.S. = not significant.

some representative results are given. It is remarkable that the results for spleen cells do not parallel those obtained with lymph node cells.

The specific response to MTV is not dependent on the virus strain that is used as antigen. A representative experiment is shown in Table 6; repeated tests yielded similar results.

Also in this assay the fluctuation in response seemed to be related to tumour size. In Fig. 5 are plotted the values for MTV-specific stimulation against tumour weight. The data have been obtained in a very short period (1 month). To keep conditions as standardized as possible, the same antigen preparations and FCS batch were used. A similar fluctuation related to tumour-size is observed as with the LAI-assay. In various other experiments we noticed the same general pattern: peak reactivity when tumour weight is small, a strong decline with increasing tumour weight and partial recovery when tumour size is very large.

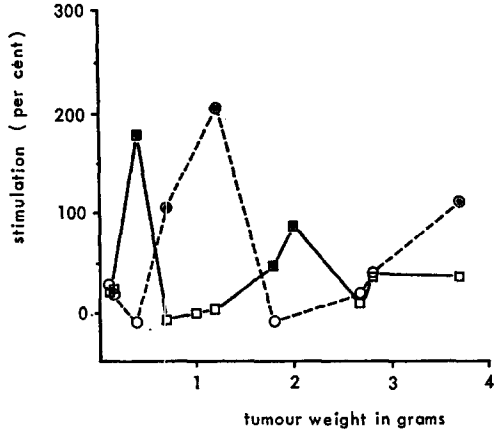

Fig. 5. MTV-specific proliferation of spleen and lymph node cells of mice of different strains bearing spontaneous tumours. All tests are performed with the same antigen preparation and batch of FCS. □: *spleen cells*; ○: *lymph node cells. A dot in the symbols indicate significant stimulation* ($P < 0·025$).

DISCUSSION

With two different techniques, the LAI and the LS test, it was found that mice bearing mammary tumour have cellular immunity to MTV. Specificity of the reaction was mainly deduced from significant differences in reactivity of the leukocytes when relatively small amounts of MTV are added as compared to the addition of similar amounts of an unrelated oncornavirus (RLV) or ovalbumin.

Both tests yielded highly reproducible results. For instance, when in the LAI test 2 microtiter plates in one experiment are compared the difference in reduction proves to be always less than 5%. In both tests it was necessary to have simultaneous controls. In our experience it is not warranted to compare results obtained at different days.

In the LAI test the plating efficiency is relatively low (from 2–10%). This is mainly due to adherence of cells to the sides of the

Table 5. Proliferation of leukocytes of mice bearing spontaneous tumours

Mouse strain	MTV-strain	Average tumour weight (±S.E.)* (g)	Leukocytes derived from	Counts/min ± S.E. in medium control	Counts/min ± S.E. in the presence of RLV	Counts/min ± S.E. in the presence of MTV	Significance of specific response
BALB/cfC3H	MTV-S	0·4 ± 0·1	Spleen	2·039 ± 901	2·492 ± 628	6·836 ± 748	$P < 0·001$
			Lymph nodes	N.D.†	2·829 ± 280	2·022 ± 319	N.S.‡
		0·8 ± 0·3	Spleen	N.D.	6·671 ± 811	11·793 ± 397	$P < 0·005$
			Lymph nodes	N.D.	4·598 ± 630	5·982 ± 241	N.S.
		1·0 ± 1·0	Spleen	4·837 ± 931	4·926 ± 843	4·959 ± 1·312	N.S.
			Lymph nodes	N.D.	8·973 ± 1·310	27·694 ± 688	$P < 0·010$
		1·4 ± 0·3	Spleen	N.D.	3·875 ± 289	4·939 ± 296	N.S.
			Lymph nodes	N.D.	2·880 ± 670	9·234 ± 738	$P < 0·010$
BALB/c	MTV-O	0·1 ± 0·1	Spleen	6·067 ± 242	14·389 ± 936	17·694 ± 1·130	N.S.
		3·7 ± 1·2	Lymph nodes	N.D.	3·274 ± 621	7·195 ± 371	$P < 0·001$
GR	MTV-P	0·2 ± 0·1	Spleen	4·368 ± 361	14·709 ± 713	16·998 ± 1·998	N.S.
		0·7 ± 0·2	Lymph nodes	N.D.	4·876 ± 1·240	9·972 ± 1·900	$P < 0·025$

*S.E. = standard error. †N.D. = not done. ‡N.S. = not significant.

wells. The stimulation of leukocyte blastogenesis by MTV is relatively low (2–4 times the background) as compared to stimulation by plant lectins. Similar low values have been found in the mixed leukocyte-tumour cell interaction test for a virally induced rat lymphoma [20].

In the present study, there seems to be a good agreement between the LAI and the LS tests, although cells from the same animals have not been compared in both assays. The difference in behaviour between lymph node and spleen cells in the LS test is interesting. This has also been found with the MC test for the B16 mouse melanoma [21]. It must be taken into account that both organs harbour different subpopulations of T cells [22, 23].

The virions of the different MTV-strains show great serological differences, as detected by heterologous antisera [24–26]. By means of blocking experiments, Blair *et al.* [16] found additional indications for type-specificity in the MC test. In the LAI and LS tests, the reaction seems to be mainly group-specific. This may be due to the fact that different subpopulations of leukocytes are involved in the various test systems.

A discrepancy between the results from this study and those obtained with the MC test [15], and those with the macrophage migration inhibition test [17], is that antiviral cell-mediated immunity estimated with the two techniques used here is demonstrable in tumour-bearing mice only, as has also been found for the BALB/cfC3H line by Sigel *et al.* [27]. Most likely, the various techniques employed measure activity of different subpopulations of leukocytes.

Sigel *et al.* [27] reported that using the leukocyte migration inhibition assay and blastogenic transformation test, positive reactions were obtained with MTV-containing milk in cells from uninfected BALB/c mice. They assumed this to be due to activation of an endogenous gene coding for an MTV protein. In our tests normal BALB/c mice were unresponsive, but this may be due to the young age of the mice in our studies.

In both tests highest reactivity was found when the animals had small tumours. This indicates that a relation exists between cellular immunological reactivity and antigenic load, although tumour weight is only a rough estimate of the latter. Also from other animal studies [15, 20, 21, 28] and from clinical observations in breast cancer [29] it is known that immunological reactivity is generally impaired with increasing tumour burden

Table 6. Proliferation of leukocytes from tumour-bearing mice in the presence of different MTV strains

Mouse strain	MTV strain	RLV	Counts/min ± S.E.* Homologous MTV-S	Autologous MTV
BALB/c	MTV-O	3·003 ± 938	6·242 ± 1·038	6·276 ± 1·234
		2·403 ± 273	4·881 ± 139	5·029 ± 460
GR	MTV-P	1·751 ± 714	3·743 ± 324	3·380 ± 138

*S.E. = standard error of triplicate cultures.

Diminished reactivity against tumour-specific antigens, as revealed by a reduced transplantation resistance [30] may be associated with the general immunological impairment.

After the initial peak in reactivity at low tumour weight, an almost complete loss of activity was found; however, in animals with very large tumours (above 3 g) the activity reappeared again, but at a lower level than was found with cells from animals with small tumours. Thus, cellular reactivity seems to fluctuate. In rats transplanted with the Walker 256 tumour, a cyclicity in circulating plasma opsonic activity was observed [31] and, probably as a consequence, also in phagocytic activity of macrophages. Cyclic activity of macrophages in the processing of viral antigens may be the cause of a fluctuation in antiviral cellular immunity in the MTV system.

An explanation for the decrease in cellular responsiveness to MTV in both tests may be the saturation of receptors of activated leukocytes with increasing amounts of viral antigens released into the blood stream (32). In the presence of the excess antigen, new populations of leukocytes will be activated, but this process will never keep pace with the antigenic load.

A second possible explanation is the occurrence of suppressor cells which inhibit the response of the otherwise reactive cells to MTV. Glaser *et al.* [20] have produced evidence that in the spleens of rats with a progressively growing transplanted lymphoma suppressor cells occur which inhibit the induction of leukocyte blastogenesis by Concanavalin A or the mixed leukocyte-tumour cell reaction. This latter possibility will be dealt with in the accompanying paper.

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The Effects of R 17934 (NSC 238159), a New Antimicrotubular Substance, on the Ultrastructure of Neoplastic Cells *In Vivo**†

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Abstract—*Ultrastructural investigations on 3 experimental neoplasms and on 1 human malignancy show that the* in vivo *antitumoral activity of R 17934 can be explained by its antimicrotubular properties. The induced disappearance of microtubules results in the disorganization and necrosis of dividing and non-dividing cancer cells. Mitotic normal cells (e.g., in the intestinal crypts) seem to be equally sensitive to the antimicrotubular action of R 17934, as malignant cells. Interphase normal cells however are much more resistant than their neoplastic counterparts.*

The lysosomotropic properties of the compound, when given as a micronized suspension, ensure a slow release effect and a local accumulation of the compound which can be favourably exploited in the treatment of malignant effusions.

INTRODUCTION

In a previous paper we have shown that the effects of R 17934, a new experimental antitumoral substance, on tissue cultured cells can be explained by its antimicrotubular properties [1]. It induced the almost immediate disappearance of microtubules from dividing and non-dividing cells in culture. This resulted in the complete disorganization of mitotic cells followed by necrosis or endopolyploidization.

The normal topographical distribution of several cellular organelles and the general cellular polarity was lost in interphase cells too. These effects were identical to those produced by the antimicrotubular plant alkaloids (colchicine, vinblastine, vincristine) as was the appearance of filament bundles and annulate lamellae after prolonged treatment. Since then it has been shown that the compound inhibited the formation of microtubules from tubulin in a cell-free system and that it shared the same binding site on tubulin with colchicine [2].

The aim of the investigations reported here was to see whether the reported antitumoral activity of R 17934 *in vivo* [3, 4] could be correlated with the mechanism of action on tissue cultured cells: disintegration of the microtubular apparatus.

On account of its low aqueous solubility the compound was injected as a micronized suspension. As our previous *in vitro* experiments were performed with a solution in DMSO (dimethylsulfoxide), we have investigated the distribution and activity of the compound as a micronized suspension in the tissue culture system.

MATERIAL AND METHODS

Cell culture

The non-transformed (MO) and transformed mouse embryonal cell line (MO_4) and routine culture methods are described in a previous paper [1].

In short, MO cells show an epitheloid character and form transparent sheets of

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polygonal cells at confluency. They show "contact inhibition" of movement and mitosis. The MO_4 cells were derived from MO cells by transformation with the Kirsten strain of murine sarcoma virus [5]. They lack "contact inhibition" of movement and mitosis and form multilayered cultures of pleyomorphic cells. Unlike MO cells the MO_4 cells produce invasively growing fibrosarcomas upon injection in the syngeneic host (C3H mice). Culture was done in Eagle's minimal essential medium (EMEM) supplemented with non-essential amino acids and 10% fetal bovine serum. Passage was done by routine trypsinization.

Cultures of spleen cell suspensions were set up as follows: spleens were dissected from DBA/2 mice that had received 10^5 viable L1210 cells by the intravenous route 5 days previously. The spleens were cut with scissors on a sterile nylon gauze stretched over a sterile recipient. The cells were flushed through the gauze with culture medium. Two millilitre cultures containing 10^6 viable cells/ml were set up in sterile plastic cell culture tubes (Falcon Plastics, Oxnard, California) in EMEM (as above) with 10% fetal bovine serum.

Experimental neoplasms

The strains of L1210, P388 and Lewis Lung cells were obtained from Dr. Atassi [3]. They are routinely transplanted in our laboratory in DBA/2 mice (L1210, P388) or C57BL mice (Lewis Lung).

Phase contrast observation

For observation with an inverted phase-contrast microscope (Reichert-Biovert, Bodson, Luik, Belgium) and for cell counting in quantitative experiments, the cells were seeded in plastic Petri dishes (Falcon Plastics) of 6-cm dia. For time-lapse microcinematography, the cells were seeded in culture chambers consisting of 2 coverglasses on a steel frame.

Ultrastructural observation

For ultrastructural observation, the cells were cultured in plastic Petri dishes of 6-cm dia. At the appropriate moment, the cells were rinsed twice with 0·9% NaCl solution at room temperature and were fixed in 5% glutaraldehyde in 0·1 M sodium cacodylate (pH 7·4) at room temperature for 15 min. Postfixation was done in a mixture containing 4% glutaraldehyde in 0·1 M sodium cacodylate and 1% osmium tetroxide in 0·05 M veronal acetate for 1 hr at 0°. After being stained with 0·5% uranyl acetate (40 min), the cultures were rinsed with sodium cacodylate buffer supplemented with 0·22 M sucrose (minimum 1 hr), dehydrated in a graded series of ethanol, and embedded in epon in the Petri dishes. After polymerization at 50°C (48 hr), the plastic dish was removed and cells were selected by observation with the phase-contrast microscope. The cells were either re-embedded in flat molds in epon for transverse sectioning or a hardened epon capsule was glued to the opposite site for flat sectioning.

Tissue blocks and cell pellets were processed as follows. Fixation was in 3% glutaraldehyde in cacodylate (0·1 M; pH 7·4) for 2 hr at room temperature followed by washing in cacodylate supplemented with 7·4% sucrose overnight at 4°C. Postfixation was done with 2% osmium tetroxide in veronal acetate (0·05 M; pH 7·4 with sucrose 7%) for 1 hr at 4°C. This was followed by a 5-min wash at 4°C in veronal acetate (0·05 M; pH 7·4 with sucrose 7%) and impregnation with uranyl acetate 0·5% in veronal acetate (0·05 M; pH 5·2) for 40 min at 4°C. The blocks were dehydrated in an ethanol series. This was followed by substitution with propylene-oxide (2 × 10 min); mixture propylene oxide-epon (1/1) overnight; and epon for 6 hr. Embedding was done in epon which was polymerized at 50°C during 2 days.

Semi-thin sections (± 2 μm) were stained with toluidine blue for light microscopical observation. Thin sections (± 100 nm) were stained with uranyl acetate and lead citrate and observed in a Philips EM 300 or EM 201 C electron microscope (MBLE, Brussels, Belgium).

For demonstration of acid phosphatase the procedure of Barka and Anderson [6] was used. After fixation with glutaraldehyde 3% at 4°C for 1 hr followed by 1 hr rinsing with cacodylate at 4°C the reaction was carried out in the dishes for 15 min at 37°C. Hereafter the cultures were dehydrated and embedded as described bove.

Experiments

For quantitative cell counting, MO cells were seeded in plastic Petri dishes at a density of 5000/cm^2. After 24 hr, some randomized fields were marked (minimum 4) and the cells were counted at a magnification of 160 ×. At this magnification, each field contains approximately 50 cells at 0 hr. Immediately thereafter, the test solutions were added and the same fields were counted again at the times indicated in the charts.

The uptake of micronized R 17934 was studied as follows. The MO_4 cells were

seeded at a normal passage density ($+5{\cdot}10^5$/75 cm^2) in 2·5 ml in Petri dishes (6 cm dia). After 24 hr the doubly concentrated solutions were added in a volume of 2·5 ml. Fixations for normal ultrastructural observation or for cytochemical purposes were done after 24 and 48 hr. The following experimental groups were taken: control (medium); R 17934 microsuspension (see below) at a final concentration of 100 μg/ml; latex beads with a mean diameter of 0·81 μm (Bacto-Latex 0·81; Difco Labs., Detroit, Michigan) diluted to the same optical density as the microsuspension of R 17934; latex beads with R 17934 at a final concentration of 1 μg/ml diluted from a stock solution in dimethyl sulfoxide (DMSO) of 5 mg/ml.

The following experiments were performed for the ultrastructural observation of experimental neoplasms *in vivo*.

The L1210 (10^5) or P388 (10^5) cells were inoculated intraperitoneally in CDF_1 mice in a volume of 0·2 ml.

After 5 respectively 6 days groups of mice received an intraperitoneal injection of R 17934 micronized (or the solvent) in a volume of 0·2 ml at a dose of 160, 80 or 40 mg/kg. Three mice from each group were sacrificed after 5, 10 and 30 hr respectively. The ascitic fluid was drawn off or, after 30 hr in the treated animals, the peritoneal cavity was washed with Hank's balanced salt solution. The cells were immediately mixed with a large volume of glutaraldehyde 3% and further processed as described above. Pieces of the serosal membranes and the intestines (duodenum, jejunum, colon) were processed as described above.

For i.v. inoculation 10^4 L1210 cells and 10^4 P388 cells were injected in a volume of 0·2 ml into the lateral tail vein. After 5, respectively 6 days groups of mice received an i.v. injection of the R 17934 microsuspension (or the solvent, 0·2 ml) at a dose of 160, 80 or 40 mg/kg. Three mice per group were sacrificed after 5, 10 and 30 hr. Pieces of the spleen, liver and intestine were taken and processed for ultrastructural observation.

The Lewis Lung cells were injected into the right thigh muscle (0·1 ml of 1/50 dilution of a minced tumor). After 10 days groups of mice were treated with the solvent, or R 17934 microsuspension (160, 80 or 40 mg/kg) intravenously and sacrificed after 5, 10 and 30 hr. Samples of the tumour, and the intestines were taken and processed as above. Other mice were treated intravenously with the same dose as above after 21 days. Three mice per group were sacrificed after 6 hr. Samples of the lungs containing small metastatic nodules and intestinal pieces were taken and processed.

A human patient suffering from a gastric carcinoma developed a malignant effusion in the peritoneal cavity. A volume of 3 l. had to be drawn off weekly. R 17934 microsuspension was injected intraperitoneally at a dose of 2 mg/kg. Samples of the ascitic fluid were withdrawn before, and 24 hr after, injection, and processed as described above.

Compounds and solutions

R 17934 (methyl [5–(2–thienylcarbonyl)–1H–benzimidazol–2–yl] carbamate; MW. 301, 31; Janssen Pharmaceutica, B-2340 Beerse, Belgium) was either dissolved in DMSO (5 mg/ml) and diluted to the desired concentration or micronized according to the following procedure. The compound was mixed with the microsuspension vehicle which contained the following components for 100 ml of fluid: 1·0 g Tween 80; 2·2 g $Na_2HPO_4.12H_2O$; 0·25 g $NaH_2PO_4.H_2O$; 0·5 g Plasdone. The pH was adjusted to 7·2. The suspension was put into glass vials (100 ml) containing glass beads and rotated on an axle for 3 days at a speed of 40 rev/min. This suspension contained particles with a maximum diameter of 2 μm. The mean and minimum diameter could not be determined owing to the lower level of efficacy of the Coulter Counter (0·4 μ) (Coulter Electronics Inc., Hialeah, Florida).

RESULTS

Effects of R 17934 micronized on the growth and viability of tissue cultured cells

Treatment of MO or MO_4 cells with a micronized suspension of R 17934 produced effects identical to those obtained with a solution in DMSO [1].

The bilateral symmetry of the cells was lost within 2 hr and the cells assumed a round epitheloid character. Directional cell migration ceased and the saltatory movement of intracellular particles was replaced by cytoplasmic mass streaming which resulted in the dispersion of the perinuclear accumulation of lysosomes.

The effects on cell kinetics were identical too (Chart 1). Mitotic cells accumulated and as a result of endopolyploidization the population gradually became multimicronucleated. The dose dependence was identical with a minimal effective dose of 0·04 μg/ml.

The only difference was the apparent loss of reversibility (Charts 2–4). While the effects were almost immediately reversible when the

DMSO solution was used the micronized suspension showed irreversible effects even after 2 hr incubation only.

The ultrastructure of tissue cultured cells treated with R 17934 micronized

From the ultrastructural point of view the effects of R 17934 on MO_4 cells treated with large numbers. However, microtubules failed to reappear when the micronized suspension was washed away contrary to the DMSO solution [1].

Both extracellular crystalline structures and identical crystals enclosed in endocytic vacuoles were consistently found in the cultures treated with the micronized suspension (Fig. 1). Since

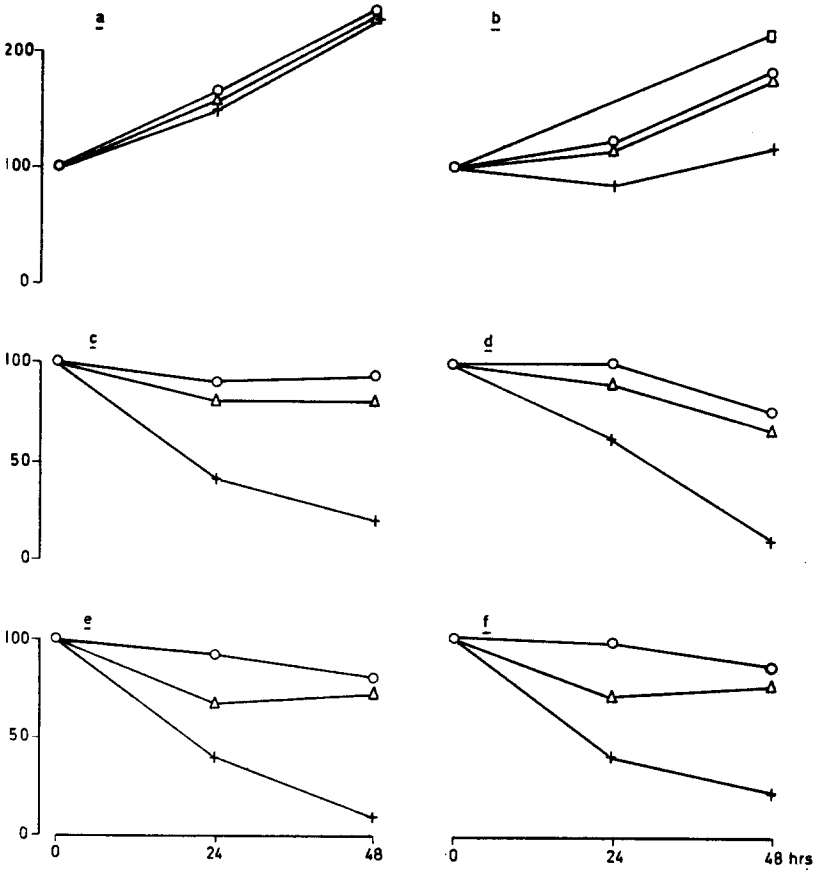

Chart 1. *Quantification of the effects of R* 17934 *as a micronized suspension on MO cultures. For technical details see "Material and Methods". R* 17934 *was added immediately after* 0 *hr. The results are expressed as percentages of the number of cells before treatment* (0 *hr*) *and the different classes are superimposed on each other,* +, *percentage of normal mononucleated cells;* Δ, *percentage of multimicronucleated cells* (*plus* [+]); ○, *percentage of mitotic cells* (*plus* [+] *and* [Δ]); *a, effect of R* 17934 (0·01 *μg/ml*); *b, effect of R* 17934 (0·02 *μg/ml*) *and total growth of the control culture represented by* □, *the different percentages in the control culture were identical to those in Chart* 1, *a; c, effect of R* 17934 (0·04 *μg/ml*); *d, effect of R* 17934 (0·1 *μg/ml*); *e, effect of R* 17934 (1 *μg/ml*); *f, effect of R* 17934 (10 *μg/ml*).

R 17934 micronized were identical to those obtained with the DMSO solution [1]. Microtubules disappeared resulting in the accumulation of aberrant mitotic cells and in the randomization of the topographical distribution of lysosomes and Golgi elements. After 24–48 hr numerous multimicronucleated cells were formed through the separate enveloping of the dispersed chromosomes. Annulate lamellae and bundles of 100 nm filaments appeared in these were never found in untreated cultures or in cultures treated with the DMSO solution these were most probably the micronized R 17934-particles. The crystalline structures appeared as empty profiles delineated by a faint flocculent precipitate. Because of their high solubility in organic solvents the original crystals were most probably dissolved by the procedure for ultrastructural preservation which includes dehydration in ethanol.

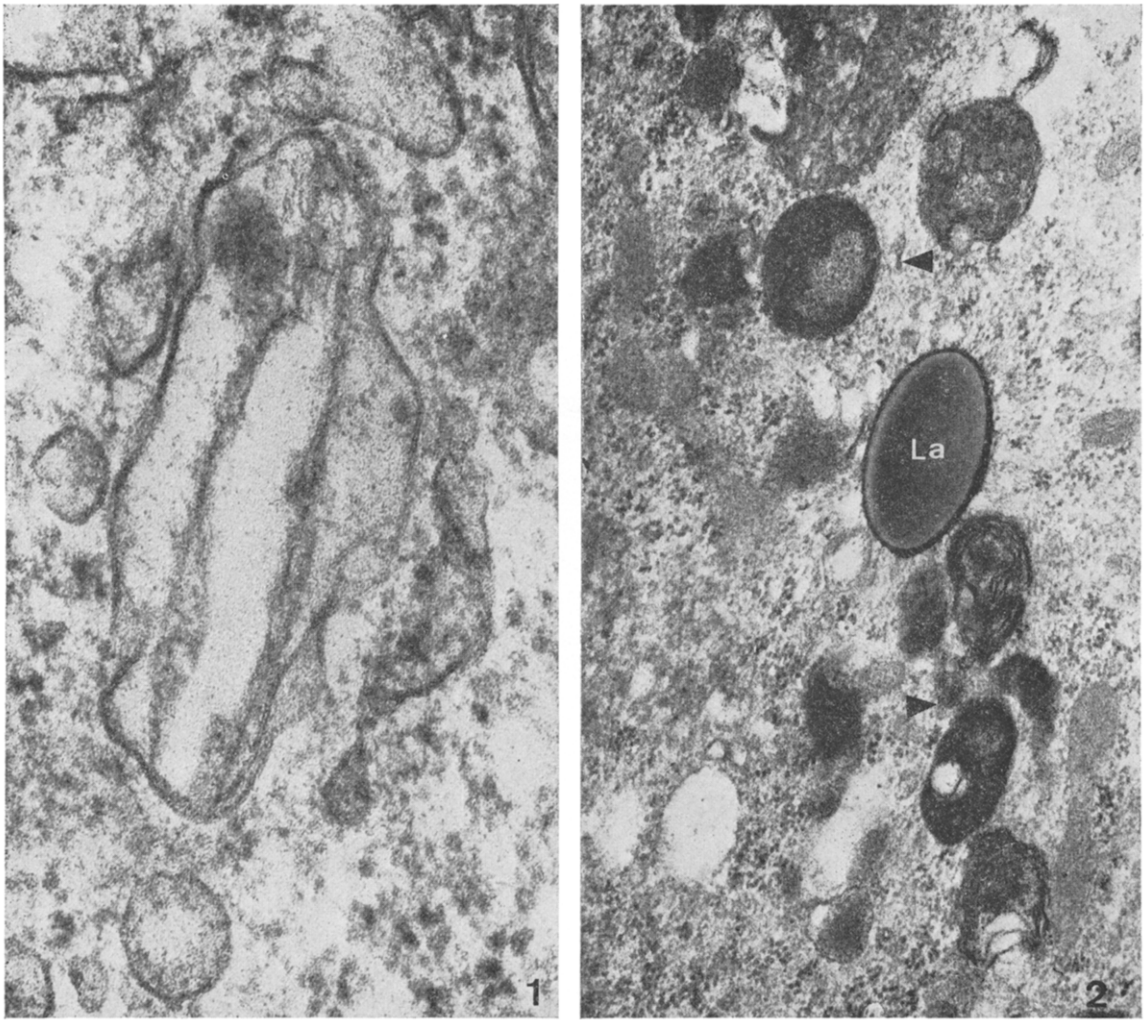

Fig. 1. *MO_4 cell treated for* 24 *hr with* 100 *μg/ml of R* 17934 *microsuspension. A vesicle with an irregular shape shows an empty inclusion the contour of which suggests a crystalline nature.* (× 34,500).

Fig. 2. *MO cell treated for* 48 *hr with* 1 *μg/ml of R* 17934 *in the presence of latex particles. A latex particle* (*La*) *is enclosed in a lysosome showing peripheral lead precipitation due to the cytochemical reaction for acid phosphatase. Several other positive lysosomes* (*arrowheads*) *with a heterogeneous content are visible.* (× 110,250).

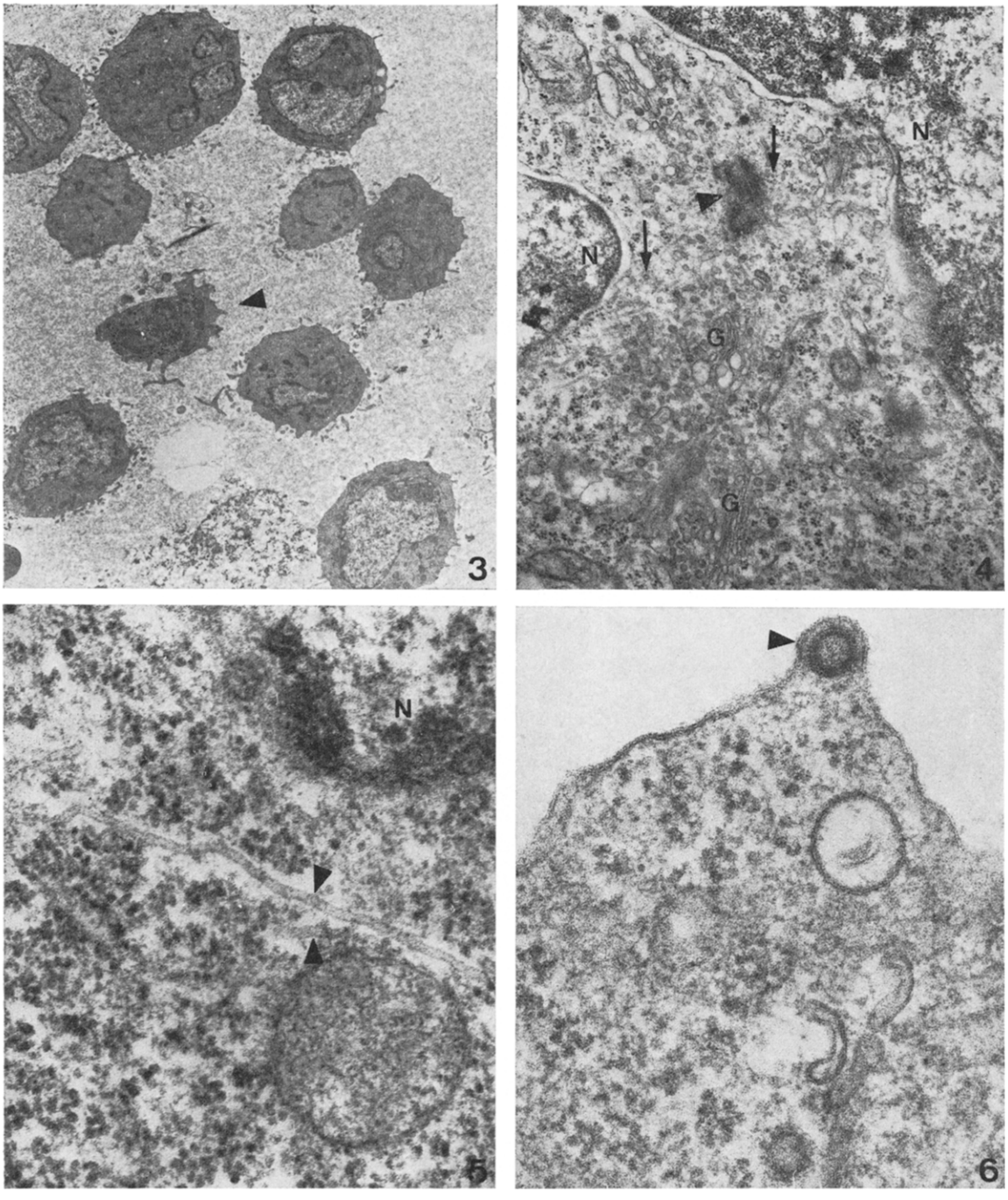

Fig. 3. *A representative sample of the ascitic fluid* 5 *days after intraperitoneal injection of P*388 *cells. Several round P*388 *cells, covered with microvilli are present. One leukocyte is visible (arrowhead).* (× 2950).

Fig. 4. *The cytocentre of an untreated P*388 *cell. The centrioles (arrowhead) surrounded by Golgi elements (G) are situated in a nuclear (N) invagination. Many microtubules radiating from the centriolar complex are sectioned transversely or longitudinally (arrows).* (× 20,000).

Fig. 5. *Large magnification of cytoplasmic microtubules (arrowheads) in the vicinity of the nucleus (N) in an untreated L*1210 *cell.* (× 69,300).

Fig. 6. *A C-type particle (arrowhead) during the stage of budding from the plasma membrane in a L*1210 *cell.* (× 100,800).

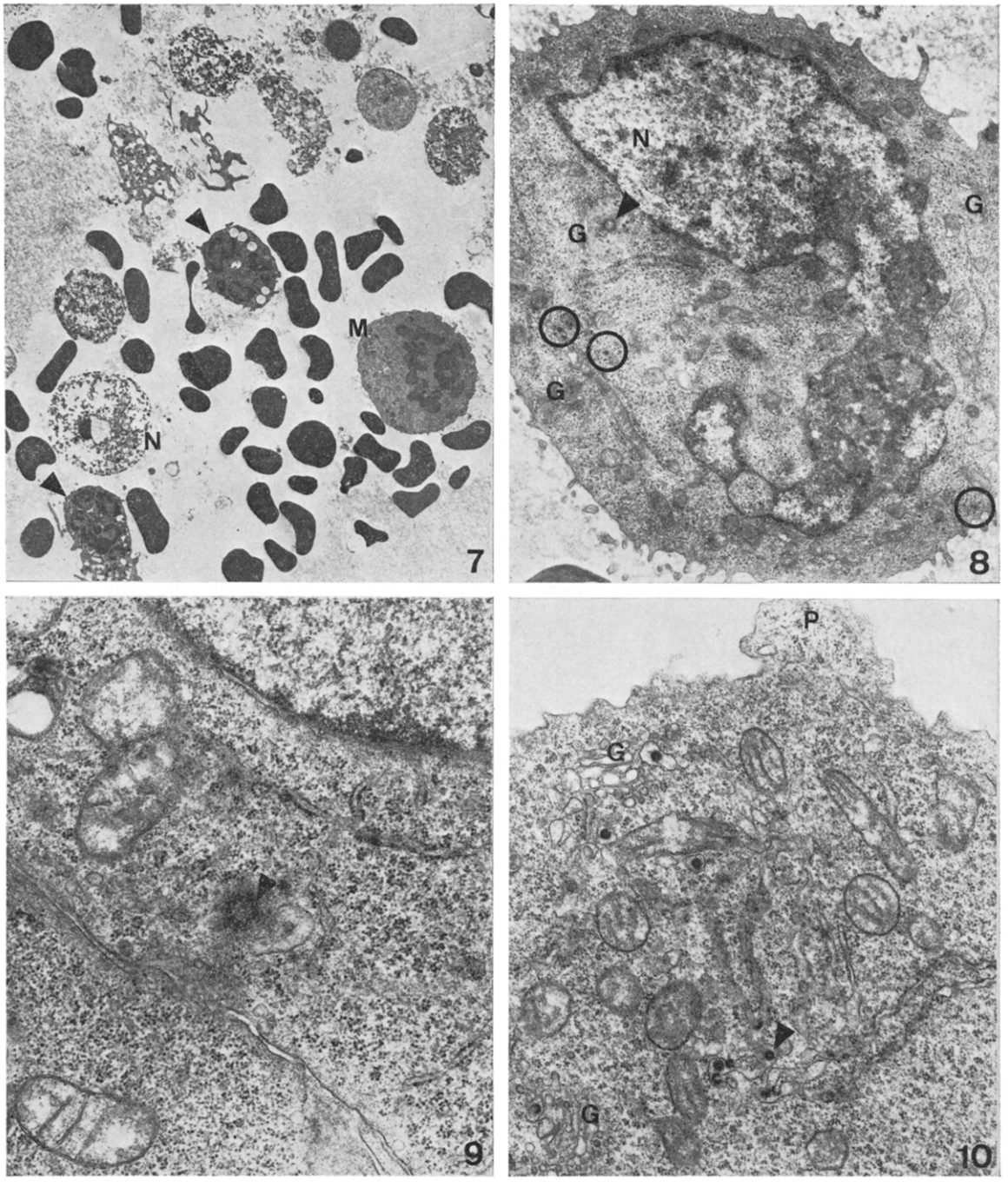

Fig. 7. *A representative sample of the ascitic fluid, produced by i.p. injection of P*388 *cells,* 30 *hr after one i.p. treatment with* 160 *mg/kg of R* 17934. *Most P*388 *cells are necrotic* (*N*). *One disorganized mitotic cell* (*M*) *and several intact leukocytes* (*arrowheads*) *and erythrocytes are present.* (× 2350).

Fig. 8. *An ascitic P*388 *cell* 5 *hr after i.p. injection of* 40 *mg/kg R* 17934. *Microtubules are completely absent. The centriole* (*arrowhead*) *is displaced from the nuclear* (*N*) *invagination. One Golgi complex* (*G*) *is still associated with the centriole but several others are not. Note the presence of several intracisternal A-type particles encircled.* (× 9100).

Fig. 9. *A Lewis Lung cell in a metastatic nodule in the lung,* 6 *hr after i.v. injection of* 80 *mg/kg R* 17934. *The centriole* (*arrowhead*) *is displaced towards the cell periphery. Golgi complexes and microtubules are completely absent.* (× 24,000).

Fig. 10. *An ascitic L*1210 *cell* 5 *hr after i.p. injection of* 160 *mg/kg R* 17934. *Microtubules are absent. Dislocated Golgi organelles* (*G*) *assume a peripheral location. Irregular protrusions* (*P*) *of the cell cortex are visible. Note the presence of numerous intracisternal A-type particles in various stages of maturation* (*arrowhead*). (× 20,700).

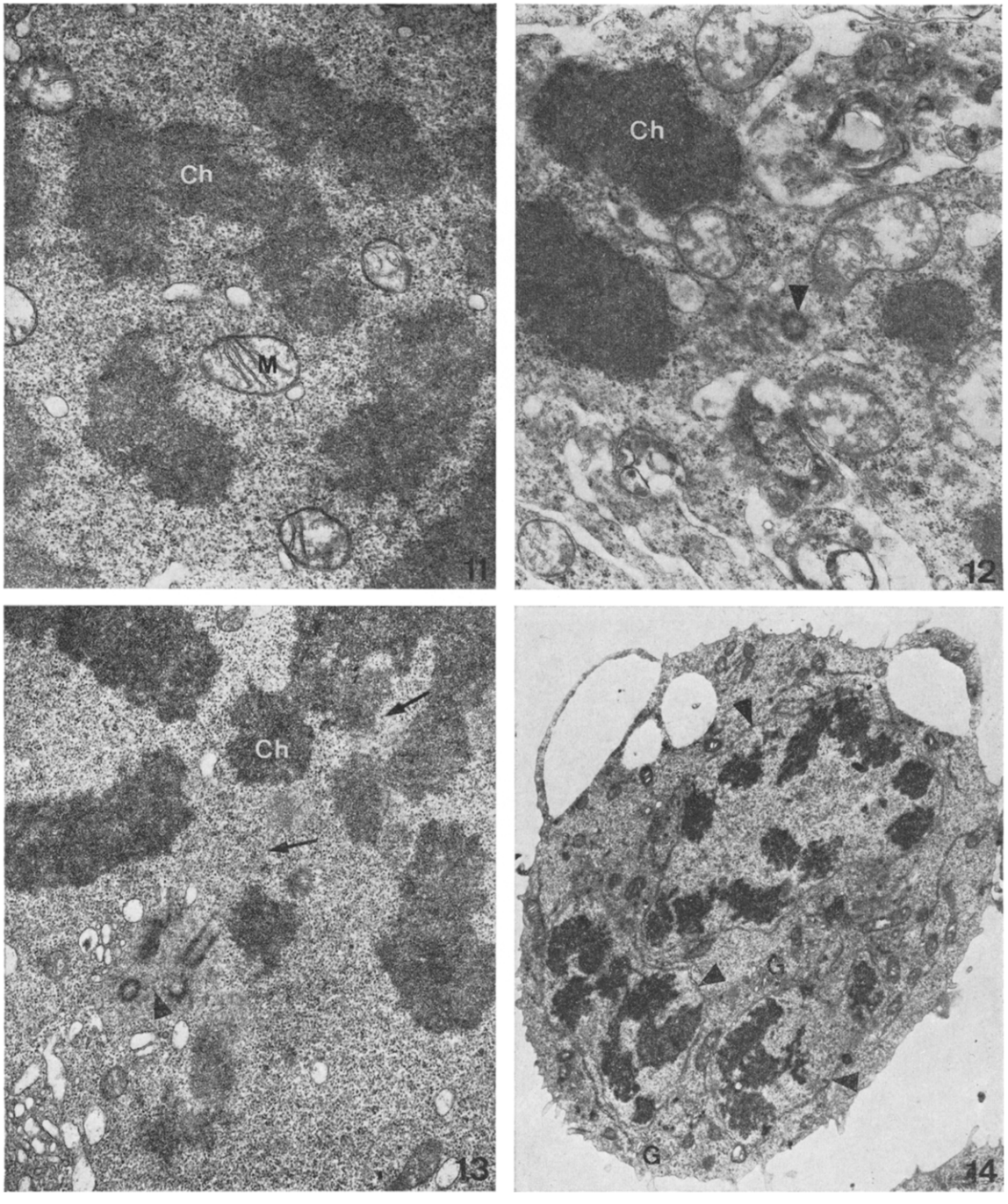

Fig. 11. *Part of a disorganized mitotic L*1210 *cell in the ascitic fluid,* 5 *hr after i.p. injection of* 160 *mg/kg R* 17934. *Spindle microtubules are completely absent. The chromosomes* (*Ch*) *are distributed in the cytoplasm without any order intermingled with other organelles such as mitochondria* (*M*). (× 21,250).

Fig. 12. *Part of a disorganized mitotic Lewis Lung cell in an intramuscular tumor* 5 *hr after i.v. injection of* 40 *mg/kg R* 17934. *Spindle microtubules are completely absent, even in the centriolar vicinity* (*arrowhead*). *The chromosomes* (*Ch*) *are randomly distributed and intermingled with other organelles.* (× 19,500).

Fig. 13. *Part of a disorganized mitotic L*1210 *cell in the ascitic fluid* 10 *hr after i.p. injection of* 40 *mg/kg R* 17934. *Two unseparated centriolar complexes* (*arrowhead*) *are present. Only few remnants of spindle microtubules are visible* (*arrows*). *A fullgrown spindle is, however, absent. The chromosomes* (*Ch*) *are clustered around the centrioles.* (× 17,000).

Fig. 14. *Disorganized mitotic L*1210 *cell in the ascitic fluid* 5 *hr after i.p. injection of* 40 *mg/kg R* 17934. *Spindle microtubules are completely absent. The scattered chromosomal masses* (*Ch*) *are beginning to decondensate. Golgi* (*G*) *complexes reappear. Endoplasmic cisternae* (*arrowheads*) *are beginning to form a new nuclear membrane around the separate groups of chromosomes.* (× 7280).

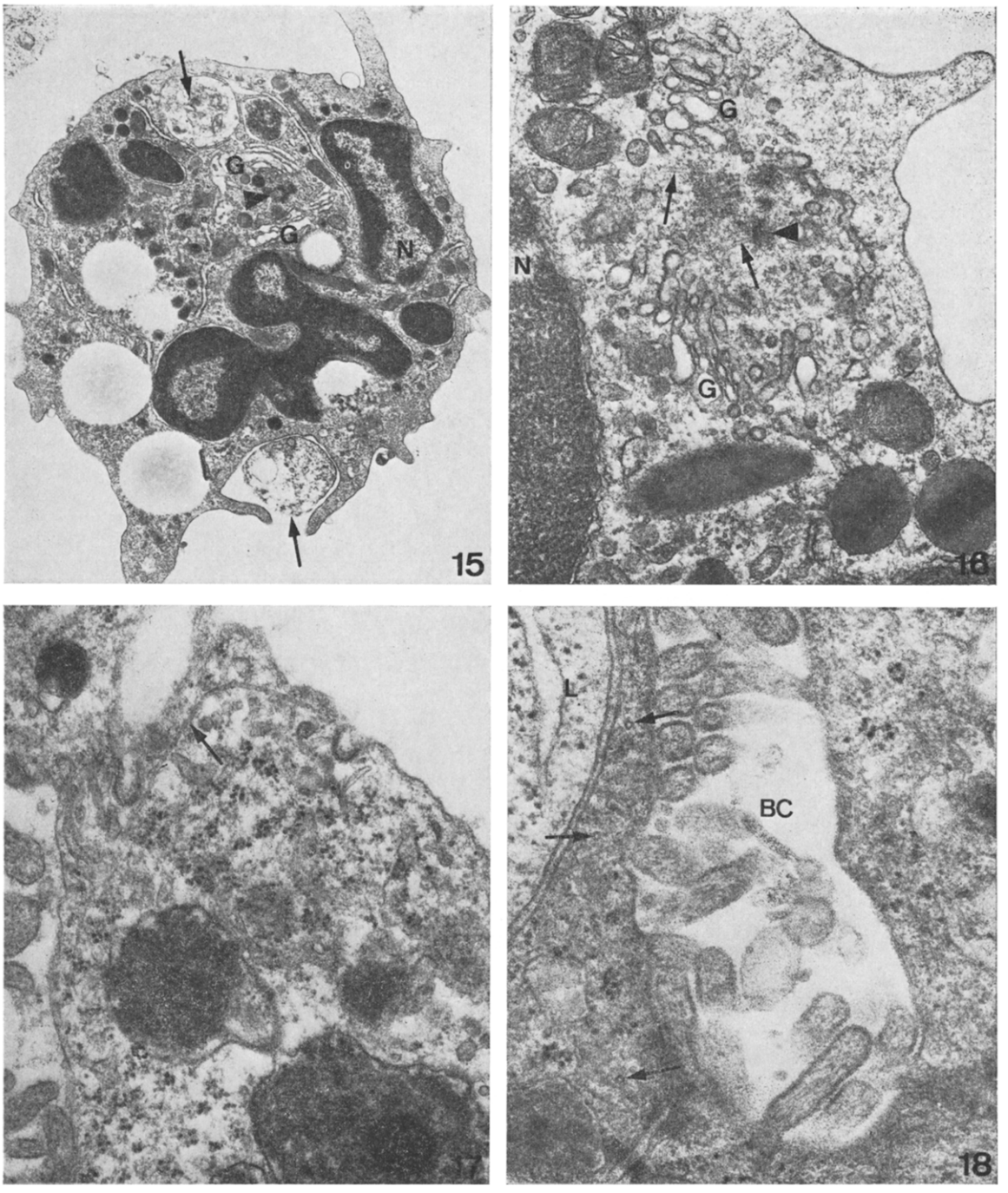

Fig. 15. *A neutrophilic leukocyte in the ascitic fluid* 30 *hr after i.p. injection of* 160 *mg/kg R* 17934. *The topographical organization of the cell is normal. Golgi complexes* (*G*) *are concentrated around the centrioles* (*arrowhead*) *in the nuclear* (*N*) *vicinity. Microtubules are present but not visible at this magnification. The cell is apparently actively engaged in the phagocytic removal of cell debris* (*arrows*). (× 12,500).

Fig. 16. *Large magnification of the cytocentre in an eosinophilic leukocyte in the ascitic fluid* 5 *hr after i.p. injection of* 160 *mg/kg R* 17934. *The Golgi complexes* (*G*) *are concentrated around the centrioles which have been sectioned through the satellites* (*arrowhead*). *Numerous microtubules can be seen originating from the centriolar satellites* (*arrows*). *A portion of the nucleus is denoted by N.* (× 37,200).

Fig. 17. *A longitudinally sectioned microtubule* (*arrow*) *in a sinusoidal Kuppfer cell* 5 *hr after i.v. injection of* 160 *mg/kg R* 17934. (× 40,000).

Fig. 18. *Several transversely sectioned microtubules* (*arrows*) *in the vicinity of a bile canaliculus* (*BC*) *in the liver* 10 *hr after i.v. injection of* 40 *mg/kg R* 17934. *Part of an invading L*1210 *cell is visible at L.* (× 46,350).

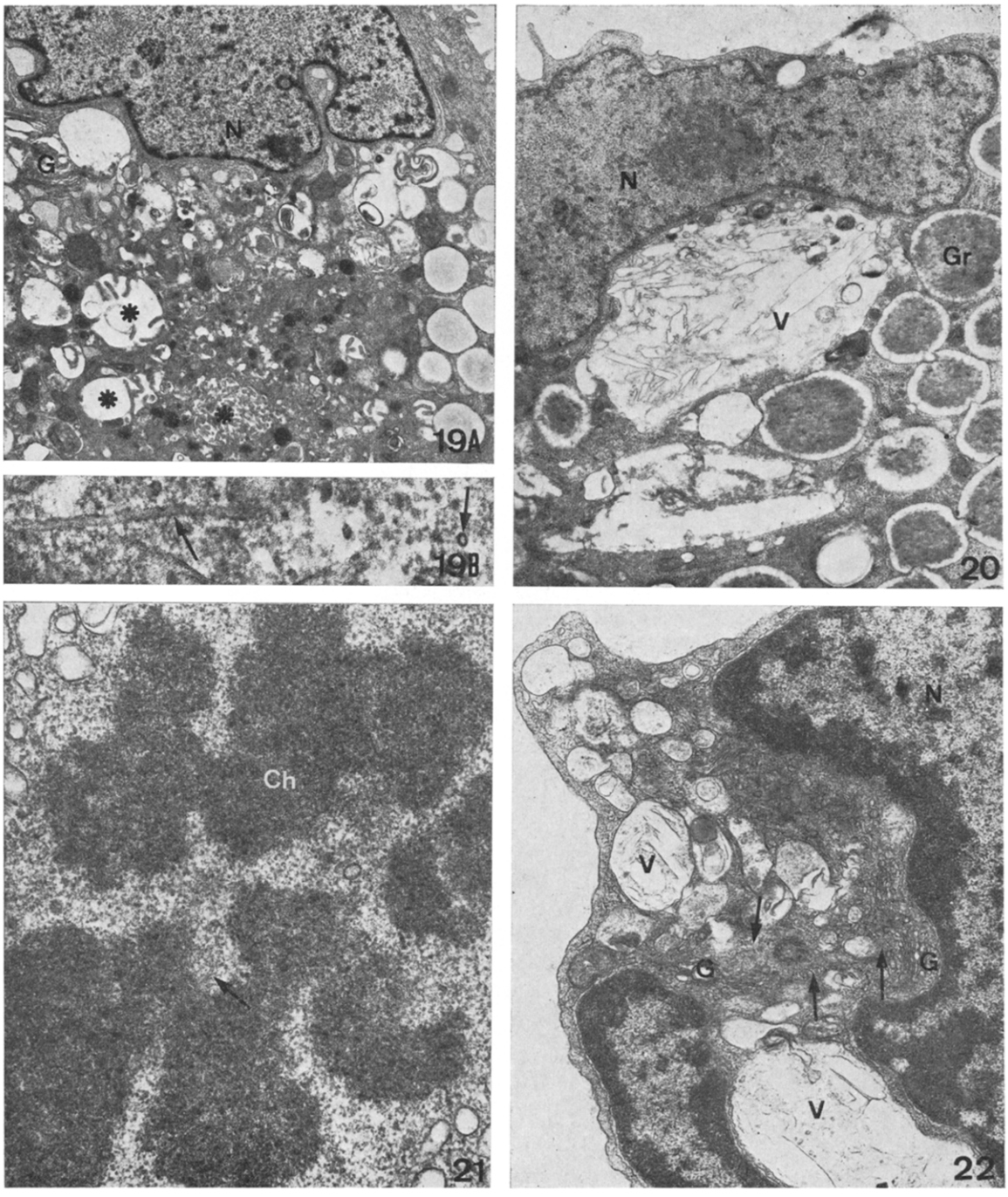

Fig. 19*A*. *Part of an untreated ascitic cell derived from a gastric carcinoma. The nucleus* (*N*) *assumes a peripheral position. Golgi complexes* (*G*) *and intracellular canalicules covered with microvilli* (*asterisks*) *are shown.* (× 6700).

Fig. 19*B*. *Transversely and longitudinally sectioned microtubules* (*arrows*) *in an untreated ascitic cell derived from a gastric carcinoma.* (× 88,200).

Fig. 20. *Part of an ascitic cell derived from a gastric carcinoma* 24 *hr after i.p. injection of* 2 *mg/kg R* 17934. *Microtubules are absent. Against the peripheral nucleus* (*N*) *a large vacuole* (*V*) *is shown containing crystalline contours. Note also the presence of numerous "secretory" granules*(*Gr*). (× 13,000).

Fig. 21. *Part of a disorganized mitotic ascitic cell derived from a gastric carcinoma* 24 *hr after i.p. injection of* 2 *mg/kg R* 17934. *The chromosomes* (*Ch*) *are randomly distributed. Spindle microtubules are absent. The arrow points to a possible microtubular remnant.* (× 25,400).

Fig. 22. *Part of a mononuclear phagocyte in the ascitic fluid, produced as a sequelae to a gastric carcinoma,* 24 *hr after i.p. injection of* 2 *mg/kg R* 17934. *Golgi complexes* (*G*) *surround the centrioles* (*arrowhead*) *located in a nuclear* (*N*) *invagination. Vacuoles* (*V*) *containing crystalline profiles are visible. Although the magnification is rather low microtubules* (*arrows*) *can easily be discerned.* (× 21,800).

It could be of interest to know whether the phagosomes containing R 17934 crystals would fuse with primary lysosomes or not in view of the known inhibitory effect of colchicine on this phenomenon in leukocytes [7] but not in macrophages [8]. This was of particular importance since R 17934 is more soluble in an acid environment such as is present in lysosomes. Unfortunately the normal cytochemical procedure used for the demonstration of acid phosphatase completely obscured the faintly visible crystal ghosts. In order to overcome this

The ultrastructure of malignant and non-malignant cells after intraperitoneal inoculation of L1210 and P388 cells and intraperitoneal injection of R 17934

The ultrastructural morphology of untreated L1210 and P388 cells is shown in Figs. 3–6.

Both cell types were more or less round with many microvilli. The cytoplasm was filled with ribosomes and a variable amount of rough and smooth cisternae of the endoplasmic reticulum (SER, RER). Some P388 cells contained annulate lamellae [9–11].

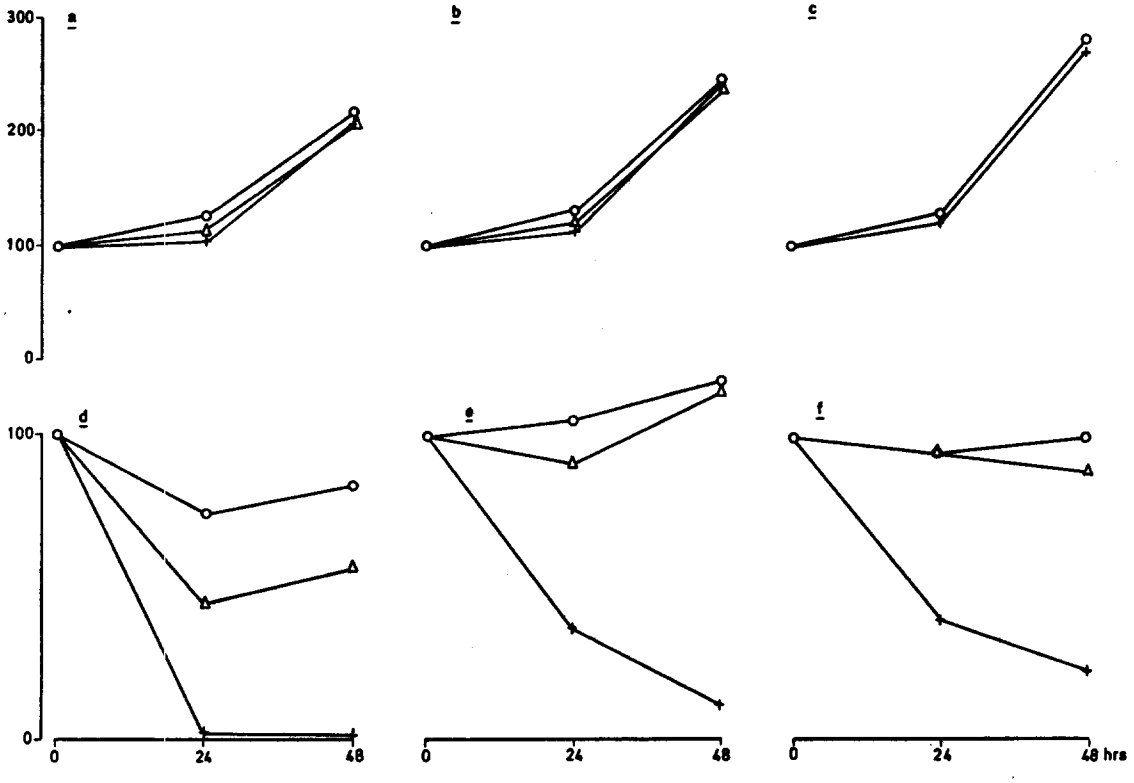

Chart 2. Reversibility of the effects of R 17934 on MO cells. The experimental set-up and symbols are the same as for Chart 1. In a, b and c R 17934 was dissolved in DMSO (5 mg/ml) and further diluted in complete culture medium. In d, e and f a micronized suspension of R 17934 in MSV (10 mg/ml) was diluted in complete culture medium.

a and d, effect of R 17934(1 μg/ml); the compound was present from 0 hr until 2 hr, then the cultures were washed twice during 10 min. and further incubated in normal growth medium; b and e, effect of R 17934 (1 μg/ml) present from 0 to 4 hr; c and f, effect of R 17934 (1 μg/ml) present from 0 to 8 hr.

we incubated the cells with latex beads of a size similar to the R 17934 particles. Both in cells treated concomitantly with a solution of R 17934 (1 μg/ml) and in untreated cells a substantial number of the engulfed latex particles was found to be enclosed in acid phosphatase-positive vacuoles (Fig. 2). Although no extensive quantitative evaluation was made the percentage of latex beads residing in acid phosphatase-positive vacuoles was even larger in the cells treated with R 17934 than in untreated cells 71% (27/38) vs 33% (39/118).

Intracisternal A-type particles were encountered in the endoplasmic reticulum of both cell types as were extracellular C-type particles which could often be seen in the stage of budding from the plasma membrane. The consistent presence of these viral particles was often of great help in excluding ambiguity with regard to the malignant origin of the cells. Lysosomes were scarce but a variable number of lipid droplets was consistently present in both cell types. The nucleus was mostly more lobulated in P388 cells than in L1210 cells where the kidney shape predominated. In both cell

types the cytocentre, formed by some Golgi cisternae surrounding the centriolar complex was located in a concave nuclear invagination. Microtubules could be seen throughout the cytoplasm. A network was present underneath the plasma membrane and a radiating complex converged towards the centrioles.

Mitotic cells showed a normal ultrastructure with a prominent spindle composed of microtubules.

intervals and with the different dosages is given schematically in Table 1.

After treatment the following observations were made on semithin sections. An accumulation of mitotic cells was visible with all doses after 5, 10 and 30 hr with a maximum of 20–50% after 10 hr (see also ref. [4]). Normal anaphase or telophase configurations, however, were completely absent. After 10 hr, and even more markedly after 30 hr, the population

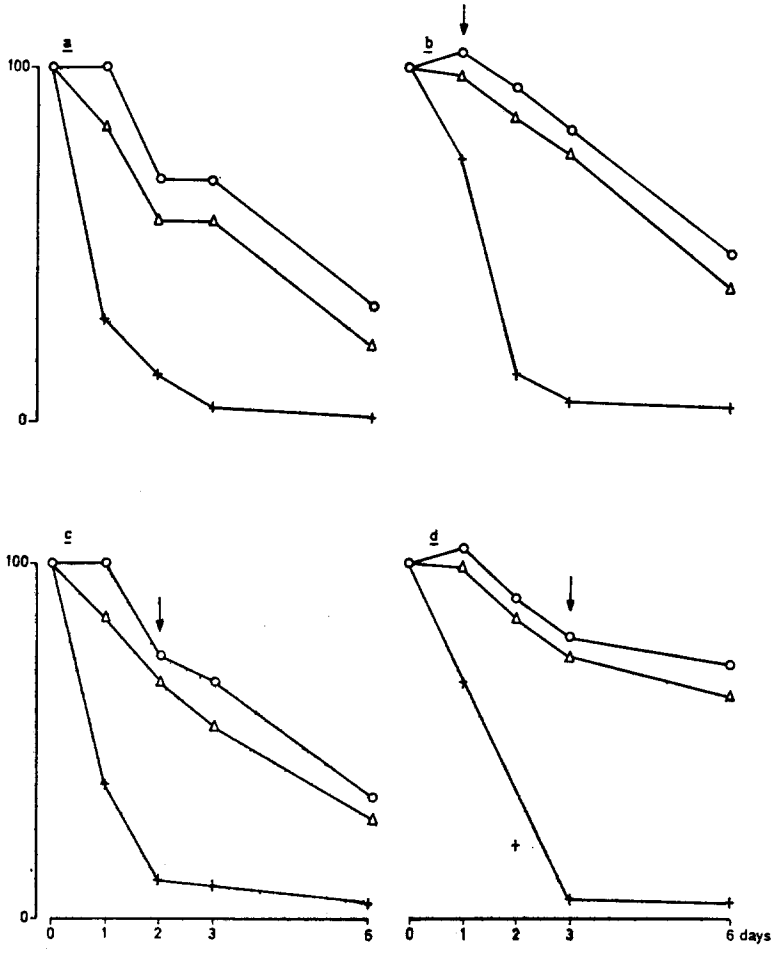

Chart 3. *Reversibility of the effects of a micronized suspension of R* 17934 (1 *μg/ml*) *on MO cultures after prolonged incubation times. The experimental set-up and the meaning of the symbols are the same as for Chart* 1. *In a, the cultures were incubated for the whole observation period with R* 17934(1 *μg/ml*). *In b, c and d, the cultures were washed (arrow) after* 24, 48 *or* 72 *hr respectively, and further incubated in normal growth medium. Control cultures had reached a plateau at* 400–600% *of the starting value after* 6 *days.*

Observation of semithin sections showed a small percentage of mitotic cells (2–5%). A small number of polymorphonuclear leukocytes was consistently present.

The effects of R 17934 on P388 and L1210 cells inoculated intraperitoneally were completely comparable. In order to avoid useless repetition a single description of the essential observations will be given. The extent of the effects in the two systems after different time

consisted predominantly of polymorphonuclear leukocytes (up to 90% after 30 hr) and some monocytes or macrophages and lymphocytes. The remaining L1210 or P388 cells were often necrotic with pyknotic chromatin.

Five and 10 hr after i.p. injection of the micronized suspension of R 17934 microtubules were largely absent from both mitotic and interphase cells at the three dosages used. Vacuoles with crystalline inclusions were most

prominent at the earliest sampling time. They were rarely seen at later intervals. The general cell shape remained round but deviating forms were encountered. The cell surface showed more blunt protrusions of various shapes than untreated cells (Figs. 7, 8, 10, 11, 13, 14).

The structures of other subcellular organelles appeared unaltered. However, the distribution of the Golgi cisternae was completely changed. They were dispersed over the entire cytoplasm and the concentration around the centriolar complex was lost (Figs. 8, 10). Annulate lamellae and bundles of 10 nm filemaents were encountered frequently after 10–30 hr.

Five and 10 hr after treatment the mitotic cells too, were completely devoid of microtubules and the chromosomes were scattered throughout the cytoplasm intermingled with other organelles. Two prominent kinetochores were often visible (Figs. 7, 11, 13, 14).

Thirty hours after treatment the accumulation of ascitic fluid had completely disappeared at all doses. The peritoneal cavity had to be washed with Hank's balanced salt solution in order to recover the remaining cells. The largest percentage of the cells consisted of eosinophilic and neutrophilic leukocytes. Some large mononuclear cells and lymphocytes were also present. The remaining L1210 or P388 cells were either in the disorganized mitotic stage or showed many signs of necrosis (Fig. 7). They contained dispersed masses of pyknotic chromatin and accumulations of membranous structures and lipid droplets. Only very few viable interphase cells were present. Some of these showed a variable number of separate nuclei. The mitotic cells

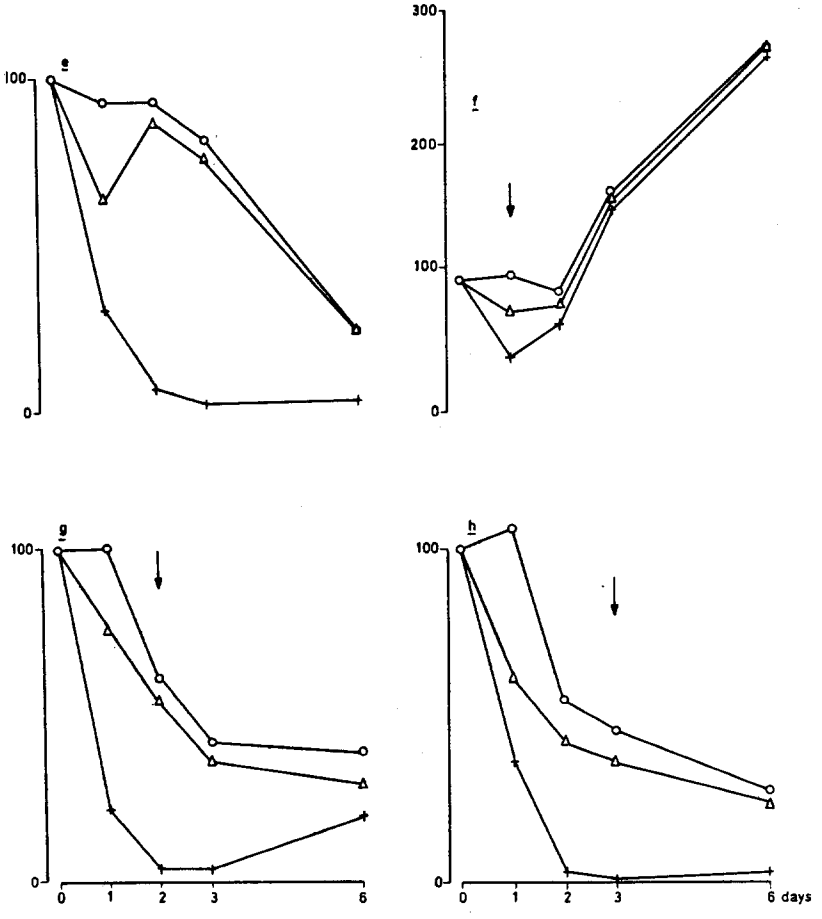

Chart 4. *Reversibility of the effects of a solution of R* 17934 (1 *μg/ml diluted from a solution of* 5 *mg/ml in DMSO) on MO cultures after prolonged incubation times.*

The experimental set-up and the meaning of the symbols are the same as for Chart 1. *In a, the cultures were incubated for the whole observation period with R* 17934 (1 *μg/ml*). *In b, c and d, the cultures were washed (arrow) after* 24, 48 *or* 72 *hr respectively, and further incubated in normal growth medium. Control cultures had reached a plateau at* 400–600% *of the starting value after* 6 *days.*

Table 1. *Effects of R* 17934 *on the microtubules in different tumor systems*

		5 hr	10 hr	30 hr
L1210 i.p.				
160 mg/kg	MT I	−	−	−+
	MT M	−	−	−
80 mg/kg	MT I	−	−+	−+
	MT M	−	−	−
40 mg/kg	MT I	−+	+−	+
	MT M	−	−+	−+
P388 i.p.				
160 mg/kg	MT I	−	−	−
	MT M	−	−	−
80 mg/kg	MT I	−	−	−+
	MT M	−	−	−
40 mg/kg	MT I	−	−	+−
	MT M	−	−	−
L1210 i.v.				
160 mg/kg	MT I	−	−	−+
	MT M	−	−	−+
80 mg/kg	MT I	−	−	−+
	MT M	−	−	−+
40 mg/kg	MT I	−	−+	+−
	MT M	−+	−+	+−
P388 i.v.				
160 mg/kg	MT I	−	−+	+−
	MT M	−	−	−
80 mg/kg	MT I	−	−+	+−
	MT M	−	−	−
40 mg/kg	MT I	+−	+−	+
	MT M	−+	+−	+−

		5 hr	6 hr	10 hr	30 hr
Lewis Lung					
160 mg/kg	MT I	−	−	−	+−
	MT M	−	−	−	−
80 mg/kg	MT I	−	−	−	+−
	MT M	−	−	−	+−
40 mg/kg	MT I	+−	+−	+−	+
	MT M	+−	+−	+−	+−

The meaning of the symbols is as follows:

MT I: microtubules in interphase cells
MT M: microtubules in mitotic cells
−: no microtubules present
−+: extremely few microtubules present
+−: more microtubules present but clearly less than in untreated cells
+: large number of microtubules present, without any obvious difference with untreated cells.

The Lewis Lung data refer to the intramuscular tumor except for the 6 hr interval which refers to the lung nodules.

showed no normal spindle. At 40 mg/kg some cells contained microtubules mostly in the vicinity of the kinetochores and centrioles. Microtubules were mostly absent from interphase cells at 160 mg/kg. Some microtubules were present in the centriolar vicinity at 80 mg/kg and many of them were present at 40 mg/kg. The subcellular organization (e.g., Golgi distribution) was mostly disturbed even at 40 mg/kg.

The leukocytes present in the ascitic fluid contained normal microtubules at all doses

tested and at all sampling times. Even those which contained crystalline inclusions, presumably the R 17934 particles. The Golgi cisternae were always concentrated around the centriolar complex in the nuclear hof. Particularly after 30 hr many of the phagocytes contained lysosomes with cellular debris (Figs. 15, 16).

The cells lining the peritoneal cavity and the intestinal epithelial cells showed a normal microtubular apparatus and cellular substructure too.

At the light microscopic level a mitotic block (ring-mitoses) was evident in the intestinal crypts in the animals that had received 160–80 mg/kg. This was most prominent after 10 hr. After 10 hr and more so after 30 hr many cytolysomes were visible in the intestinal epithelial cells. Presumably these were remnants of mitotic cells [12].

At the ultrastructural level a normal spindle was absent although a substantial number of microtubules could be seen. However, centriolar migration and chromosome separation had mostly failed to occur. No abnormalities were seen at 40 mg/kg.

The ultrastructure of malignant and non-malignant cells after intravenous inoculation of L1210 and P388 cells and intravenous injection of R 17934

Four to five days after intravenous injection of L1210 or P388 cells, neoplastic cell nests were found abundantly in the liver, around the sinuses and in the spleen. The morphology of the cells in untreated animals was comparable to that of the cells in the ascitic fluid after intraperitoneal inoculation. Generally they were spherical and were linked to each other and to other cells by interwoven microvilli.

The presence of multiple A-type and C-type particles was of great help in determining the nature of the cells, especially in the spleen.

Examination of semithin sections showed an accumulation of mitotic cells at all time intervals and at all doses, reaching a maximum after 10 hr, in treated animals. Normal anaphase or telophase configurations were completely absent.

At the ultrastructural level the effects of R 17934 were identical to those described for the cells in the ascitic fluid. The minor differences in time and dose dependency are shown in Table 2. Microtubules disappeared both from mitotic cells and interphase cells. This resulted in a similar disorganization and necrosis as described above.

Intracellular crystals were found after 5 hr only. Similar crystals were found in the Kupffer cells and phagocytic cells in the spleen. Microtubules however, were consistently present in these cells and other cells in the immediate vicinity of the neoplastic cells (liver parenchyma, vascular endothelial cells, splenic leukocytes etc.) at all time intervals and at all dose levels (Figs. 17, 18). However, a C-mitotic block was evident in the intestinal crypts at 160, 80 and 40 mg/kg. And a progressive accumulation of lipid droplets in the liver parenchyma was noted, reaching a maximum after 30 hr.

Table 2. Number of sections through L1210 cells and normal leukocytes with (+) or without (−) visible microtubules after a 5 hr-period of treatment in vitro *with different doses of R 17934. The [%−] gives the number of cells without microtubules as a percentage of the total number of cells. For technical details see "Material and Methods"*

	L1210			Leukocytes		
	+	−	%−	+	−	%−
Control	62	23	27	28	34	55
0·04 μg/ml	50	5	9	16	13	45
0·08 μg/ml	31	10	24	10	13	57
0·16 μg/ml	37	9	20	18	21	54
0·32 μg/ml	10	25	71	14	12	46
0·64 μg/ml	16	41	72	18	13	42
1·28 μg/ml	12	55	82	13	15	54

The ultrastructure of malignant and non-malignant cells after intramuscular inoculation of Lewis Lung carcinoma cells and intravenous injection of R 17934

The Lewis Lung cells were round with a large irregular nucleus. A variable amount of mitochondria, endoplasmic reticulum, lysosomes and lipid droplets was present. A small number of viral particles could be seen (A- and C-type). The cytocentre (Golgi cisternae surrounding the centrioles) was present in a perinuclear location. Microtubules could be found throughout the cytoplasm and were most prominent in the vicinity of the centrioles. Mitoses were completely normal.

Some infiltrating leukocytes were present and a variable number of necrotic cells especially in the hemorrhagic centre of the tumor.

The findings after treatment were similar to those in L1210 or P388 cells and will not be repeated (Figs. 9, 12). The exact time-dependency and dose-dependency are given in Table 1. The effects on the host tissues were as described above.

The ultrastructure of ascitic carcinoma cells after intraperitoneal treatment of the patient with R 17934

Before injection of R 17934 the ascitic fluid contained a large number of round cells with many microvilli. In most cells the nucleus was displaced towards the periphery. A central Golgi area was often present. The cytoplasm was filled with large granules containing an amorphous material comparable to the secretory granules of gastric parietal cells [13]. In many cells intracellular canaliculi covered with microvilli were present. Microtubules were present throughout the cytoplasm. Only a few mitotic cells were seen and some leukocytes (Figs. 19A, 19B).

Treatment with R 17934 resulted in the total disappearance of microtubules from both interphase and mitotic cells (Figs. 20, 21). This resulted in the accumulation of disorganized mitoses that became necrotic showing pyknotic chromatin. Many interphase cells too were apparently lysed. A consistent finding was the fusion of the secretory granules into large iregular masses. Several cells contained vacuoles with crystalline particles (Fig. 20).

The leukocytes contained normal microtubules and a normal intracellular organization (Fig. 22). More particularly, the Golgi cisternae remained concentrated around the centrioles in the nuclear hof.

Further treatment of the patient with 2 mg/kg every 3 days resulted in the gradual disappearance of the ascitic fluid. Regular examination of smears from the fluid showed a further accumulation of mitotic cells and a gradual disappearance of malignant cells.

Ultrastructure of malignant and non-malignant cells after treatment with a solution of R 17934 in vitro

Apparently there was a clear-cut difference in sensitivity towards the antimicrotubular action of R 17934 between the malignant and non-malignant cells. This could be due to a difference in uptake of the micronized R 17934 particles and/or a different intracellular solubilization. One other possibility is an intrinsic difference in the sensitivity of the microtubular apparatus.

In order to test the second hypothesis the spleens of animals inoculated 5 days previously with L1210 cells, were filtered through nylon gauze. The cell suspensions were put into short-term culture, and treated with different doses of R 17934 dissolved in DMSO. The L1210 cells could be distinguished from non-malignant cells (among other morphological characteristics) by the presence of the A-type and C-type particles. Normal leukocytes and L1210 cells (in interphase) were scored for the presence or absence of microtubules. The results are given in Table 2. It should be noted that the effect of R 17934 on L1210 cells is a clear-cut underestimation because cells with only 1 microtubular profile were scored as positive. This was mostly the case with the higher dosages. Normal leukocytes on the other hand showed an equal amount of microtubules with all doses. The effect on microtubules in mitotic L1210 cells was complete at 0·04 μg/ml.

DISCUSSION

The *in vitro* experiments have shown that the activity of R 17934 is not altered when the compound is administered to the cells as a micronized suspension. Most probably a minimal amount is freely soluble in aqueous media. In view of the biological activity this amount is certainly larger than 0·04 μg/ml, the minimally effective dose. Apparently the micronized particles are phagocytozed by neoplastic cells *in vitro* and *in vivo* as well as by normal phagocytes.

The immediate reversibility of the activity when the compound is solubilized and the failure of reversibility when the compound is given as a suspension show that at least part of the intracellular store can be solubilized. This is substantiated by the observed gradual disappearance of the crystals with time *in vitro* and *in vivo*.

Since fusion of the crystal-loaden phagosomes with primary lysosomes does occur, the micronized form of R 17934 can be regarded as a true lysosomotropic substance [14]. Apart from the theoretical advantages put forth by Trouet (accumulation in mostly actively phagocytozing cancer cells) the following facts can be noted. The intracellular storage ensures a slow release effect that seems to be essential for the efficacy of R 17934 *in vivo*. Indeed, unpublished observations of ourselves and Atassi [3, 4] have shown that a solution of R 17934 injected *in vivo* must be given for at least 24 hr, with 3 hr intervals, in order to obtain a minimal activity in the L1210 system.

The local accumulation of the compound makes it ideally suitable for the treatment of malignant effusions which can be achieved in human patients without any toxic side-effect (J. De Crée, personal communication).

Although intravenous injection of the micro-

nized suspension seems to be well tolerated, by mice, we have noted severe disturbances of the blood coagulation system in dogs. This precludes, for the moment, further investigations in human patients, with regard to the systemic application, until a more suitable formulation is found.

The ultrastructural investigation on 3 different experimental tumor systems and in 1 human case have shown that the antimicrotubular activity which was evident in cell culture is also apparent *in vivo*. The antitumoral activity is thus most probably related to this activity.

It is probably worth stressing that, as for the vinca alkaloids [15], the antitumoral activity of this compound cannot entirely be explained by the oversimplifying term "mitotic block". The total disorganization of non-mitotic cells should strongly affect all the vital processes which depend on ordered subcellular movements or on an adequate compartmentalization. The interference with directional cell migration could have important consequences for the invasive and metastatic properties of tumoral cells. This could explain the strong activity against Lewis Lung metastasis [3]. At last, both the alteration of the fluid mosaic behavior of the cell membrane [16] and the altered turnover of the cell coat [17] could seriously change the interaction with immune and non-immune host defense mechanisms. In view of this, the apparent difference in sensitivity between interphase malignant and non-malignant cells towards R 17934 could be a favorable factor in determining its therapeutic efficacy.

Apparently this difference in sensitivity is not related to a differing capacity to concentrate the micronized particles since it could be reproduced by using a solution of R 17934 *in vitro*. Theoretically several possibilities remain open. The soluble drug could reach higher concentrations in neoplastic than in normal cells through differing membrane permeabilities. This should be determined with uptake studies using the radiolabeled compound. A faster metabolism of the compound by normal cells is not very likely since we have never seen any metabolization of the compound (*in vitro*) as determined by biological tests. Finally, it may not come as a surprise to learn that the microtubular apparatus of cancer cells which have retained the capacity of division is more labile than that of cells which normally no longer divide. This is substantiated by the recently observed differences between transformed and untransformed cells with regard to their cytoplasmic microtubule complex [18].

The mitotic normal cells, however, (intestinal crypts) are apparently equally sensitive to the antimicrotubular activity of R 17934 as the mitotic malignant cells.

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The Use of Macromolecules as Carriers of Antitumor Drugs

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Abstract—*The purpose of this investigation is an extension of the use of protein carriers to various types of antitumor drugs, including antimetabolites and antitumor antibiotics. The variety of carriers has also been increased by the use of branched chain synthetic polymers with a poly-L-lysine backbone. Most drug-protein combinations were non-covalent in nature. Murine antitumor results are discussed based on the evaluation of activity in the leukemia L1210 and P388 systems. In several cases the therapeutic efficacy of an antitumor drug was increased by macromolecular complexation. This effect was partly due to reduced toxicity and seems to be dependent on the choice of carrier as well as the choice of drug. Actinomycin D, cytosine arabinoside, 5-azacytidine and dichloromethotrexate appear to be good candidates for a macromolecular complex approach to cancer chemotherapy.*

INTRODUCTION

SEVERAL approaches have been made to enhance the efficacy of existing antitumor agents through the discovery of better ways to use these drugs. One successful attempt has been combination therapy, which has been receiving increasing attention. Another recent approach has been the use of large molecular weight materials as carriers of cytotoxic drugs [1, 2]. The high endocytic activity reported for several types of tumor cells might allow a preferential uptake of the drug-macromolecular combinations by the target cell. This device could serve as a means of directing drugs preferentially into a chosen cell type. Since almost any substance can be linked by suitable physical or chemical coupling methods with an appropriate carrier, this field of research offers rich possibilities for a novel kind of drug design.

It has been demonstrated that cytotoxic drugs can be covalently coupled to macromolecules without loss of biological activity [1, 3–5]. Our earlier studies of a series of products in which a nitrogen mustard derivative [*p*-(N, N-bis-2-chloroethyl)aminoaniline] was chemically attached to albumin, fibrinogen, γ-globulin or their poly-alanyl derivatives showed that this type of covalent coupling (conjugate formation) led to an improvement of up to 8 fold in therapeutic index in the ADJ/PC6A plasma cell mouse tumor screen [1]. It was further shown that covalent bond formation was not a necessity and that complex formation by physical association was adequate in many cases [2]. As a result of several studies it has become clear that the strength or stability of bonds between a carrier molecule and a drug plays a critical role in the determination of its biological properties. While covalent bonds are sufficient, they are not necessary since the cumulative energy of multiple ionic and other non-covalent bonds between two molecules may produce very stable complexes [6].

In order to assess the value of different types of protein carriers, and evaluate complexes with antitumor agents other than nitrogen mustard derivatives, a series of new protein-antitumor agent combinations were prepared and evaluated in the leukemia L1210 system. The structural formulas of the various drugs used in these experiments are shown in Fig. 1. All of the antitumor agents evaluated were of some interest to the Division of Cancer Treatment, National Cancer Institute at the time of the study. Included are a broad spectrum of antitumor agents (antibiotics, nucleosides, intercalating agents, antimetabolites). In most instances non-covalent bonding was employed (complexes). In a few cases an

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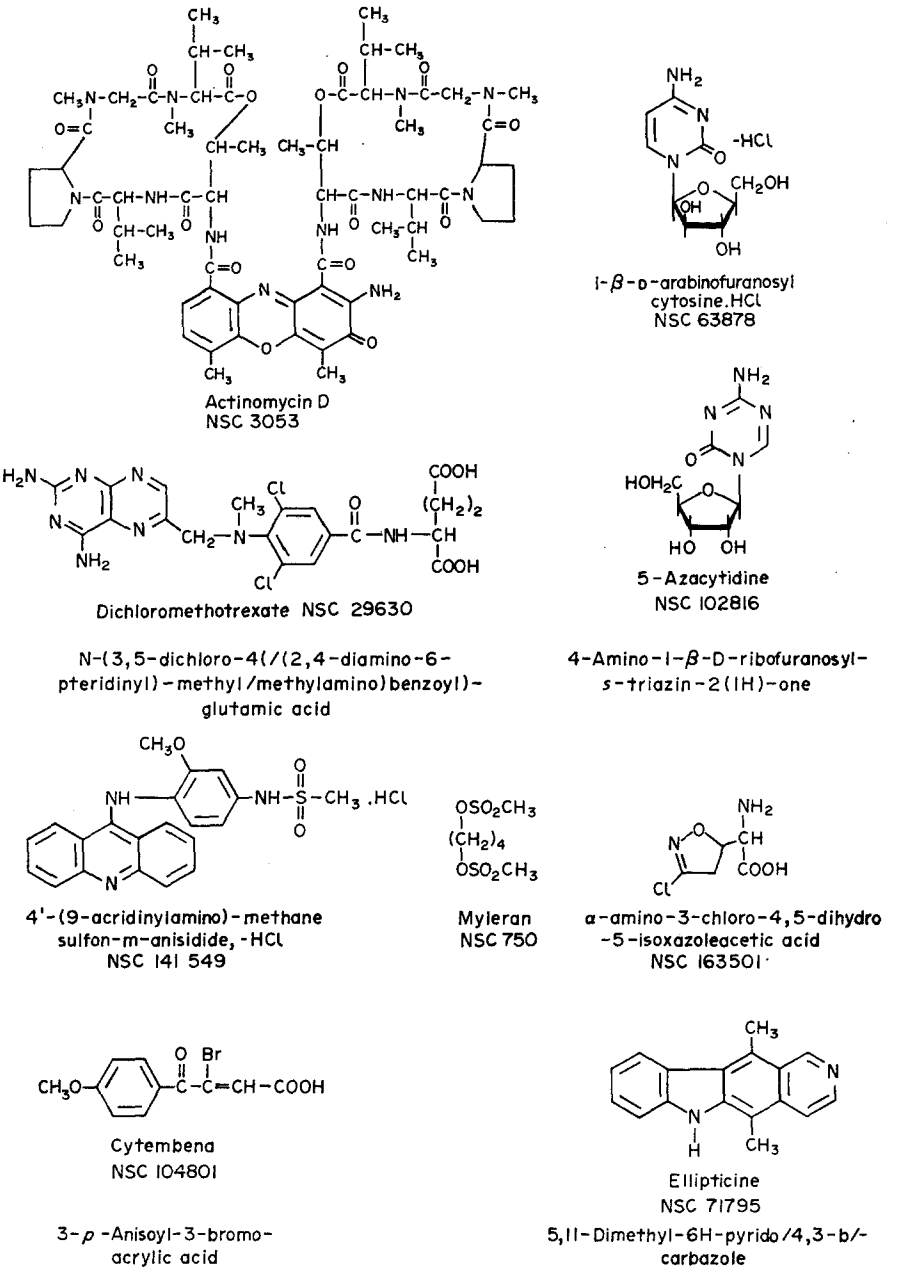

Fig. 1. Structure of antitumor drugs used for coupling to macromolecules.

attempt to form covalent bonds between drug and protein (conjugates) was made. Non-covalent linkages have the great advantage that they can be formed without the help of special chemical technology.

The number of carriers studied was increased relative to previous experiments [1, 2] by the use of synthetic multi-chain polyamino acids with a polylysine backbone. This type of material has been extensively used by Sela *et al.* [7, 8] as model compounds in immunological studies. These structures can be modified easily in many ways (charge, spatial requirements, immunological properties, etc.).

There are indications in the literature that the pinocytic activities of different cell types show some chemical specificity [9]. Our previous results showed that coupling equal amounts of the same drug covalently to different carriers gave rise to considerable variation in the tumor-inhibitory potency of the conjugates. In order to achieve cell-specific effects, additional studies are necessary in order to develop carriers which rely on selective endocytic uptake by the target cells.

MATERIAL AND METHODS

Drugs

The cytotoxic drugs used are shown in Fig. 1. All the drugs were supplied by the Drug Development Branch, National Cancer Institute (Bethesda, Maryland).

Carriers

Three groups of carriers were used. (*a*) Plasma proteins: bovine serum albumin, bovine γ-globulin, bovine fibrinogen, fraction I (Sigma). (*b*) Poly-DL-alanyl proteins. These were prepared as reported previously [1, 10]. (*c*) Synthetic polypeptides. Intermediates: N^{ε}-carbobenzyloxy-L-lysine [11], N^{ε}-carbobenzyloxy-N^{α}-carboxy-L-lysine anhydride [12], N-carboxy-DL-alanine anhydride [13], γ-benzyl L-glutamate [14], γ-benzyl N-carboxy-L-glutamate anhydride [15].

Poly-L-lysine was prepared from N^{ε}-carbobenzyloxy-L-lysine via the N-carboxy-anhydride [12]. Conditions for polymerisation were chosen to obtain a degree of polymerization of approx. 100–300 [16]. The molecular weight of N^{ε}-carbobenzyloxy-poly-L-lysine was estimated by viscosity measurements [12]. The carbobenzyloxy blocking groups were removed by HBr/acetic acid. The purified poly-L-lysine had a molcular weight of 30,000 (approx.).

Multi-poly-DL-alanyl-poly-L-lysine (pDLAla—pLys) was prepared according to Sela *et al.* [17] choosing a Lys:Ala ratio 1:3·65. Amino acid analysis of the polymer indicated a ratio Lys:Ala = 1:3·5.

For the preparation of multi-poly-L-glutamyl-poly-DL-alanyl-poly-L-lysine (pGlu-pDLAla—pLys) the method of Sela *et al.* [18] was adopted. Input amino acid ratio Lys:Glu—1:6·7. Analysis of end product gave Lys:Glu = 1:5·8.

Coupling methods

Method A [2]. A solution of the macromolecule in pH 6 phosphate buffer was incubated at 4°C with a solution of the drug. Agents which had a poor water solubility were applied in an organic solvent (Table 1). Stirring was continued for 3–4 hr in the cold. The macromolecular complex formed was purified in each case by dialysis for 36 hr against several changes of water using Visking tubing with the temperature being maintained below +4°C.

In the case of poly-DL-alanyl fibrinogen derivatives, dialysis was carried out against 0·3% sodium citrate solution. Products were isolated by freeze drying. Estimates of the drug content were based either on microanalytical data obtained for elements not present in proteins (e.g., chlorine) or by u.v. absorption measurements.

Method B. With drugs of reasonable water solubility, complex formation was achieved by mixing a solution of the macromolecule in water with an aqueous solution of a weighed amount of the drug for 30 min at 4°C followed by freeze-drying (Table 1). An advantage of this method is that no further analytical measurement is needed to estimate the drug content.

Method C. Covalent bonds were formed by the aid of a water soluble carbodiimide [1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methotoluene-*p*-sulphonate] in pH 6 phosphate buffer, as described previously [1].

Determination of antitumor activity

All *in vivo* antitumor tests were carried out at Hazleton Laboratories, Vienna, Virginia, U.S.A., using previously published National Cancer Institute protocols [19].

With the exception of cytembena and its protein combinations, all products were tested in the murine leukemia L1210 system. Cytembena and its derivatives (B 900302–B 900304) were screened in the P388 lymphocytic leukemia test system since the parent drug is inactive in the L1210 system. In each instance, the uncomplexed parent drug was tested for comparison in the same experiment. None of the macromolecular carriers by themselves inhibited tumor growth. Combinations formed which were found to have a low drug content and a relatively high parent drug optimum dose (e.g., Myleran) were not tested in the tumor model. Water was used as the injection vehicle. Tumor cells (L1210, 1×10^5; P388, 1×10^6) were injected into the peritoneal cavity of the test animals. Intraperitoneal injection of the test compounds began the next day and continued daily for a total of nine injections (QD 1–9 treatment schedule). Six mice were used to evaluate each dose level. Results are reported in percent survival time of treated animals versus untreated tumored control animals [(treated survival ÷ control survival) × 100% = T/C%]. The percent T/C values greater than 125% indicate statistically significant antitumor effects [19].

RESULTS

Results of animal screening are summarized in Figs. 2–9. Graphical presentations were constructed by plotting antitumor activity (percentage T/C) against units of drug dosage.

Attention should be called to the following points:

(*a*) It can be seen from the graphical presentations that if the actual drug content of the macromolecular combination is calculated, the optimal dose levels are almost

Table 1. *Survey of the preparation and composition of drug-macromolecular combinations*

Drug	Carrier	Method of coupling	Drug-macromolecule weight ratio used for coupling	%-age drug content of macromolecular derivative	Number of animal test
NSC 3053	BSA	B	1:149	0·66	B 900327
	pDLAla-BSA	B	1:49	2	B 900328
	pDLAla—pLys	B	1:49	2	B 900329
	pGlu-pDLAla—pLys	B	1:49	2	B 900330
	BSA	C	1:50	1·2	B 900326
NSC 63878	BSA	B	1:15·75	6	B 900333
	BSA	B	1:9	10	B 900340
	pDLAla-Fibrinogen	B	1:7·4	12	B 900334
	pGlu-pDLAla—pLys	B	1:9	10	—
	BSA	C	1:10	5·8	—
NSC 102816	BSA	B	1:9	10	B 900320
	pDLAla-BSA	B	1:9	10	B 900321
	pDLAla-Fibrinogen	B	1:9	10	B 900322
	pDLAla—pLys	B	1:9	10	B 900323
NSC 29630	BSA	A*	1:20	3	B 900318
	BSA	A†	1:5	4	—
	BSA	A‡	1:5	5·8	B 900319
NSC 141549	BSA	A§	1:4	3	—
	BSA	A†	1:10	5	B 900311
	BSA	A*	1:13·3	5	—
	BSA	A‖	1:10	8	—
	γ-Globulin	A*	1:10	10	—
	pDLAla-BSA	A†	1:5	9	B 900313
	pDLAla—pLys	A†	1:4·5	3·3	—
NSC 104801	BSA	A	1:3	5·3	—
	BSA	A¶	1:2·5	6·4	B 900302
	BSA	C	1:3	8	B 900304
	γ-Globulin	A,	1:2	6·1	—
NSC 163501	BSA	A	1:6·66	0·5	—
	BSA	B	1:12·3	7·5	B 900308
	Fibrinogen	A	1:10	2	—
	pDLAla-Fibrinogen	B	1:9	10	B 900309
NSC 71795	BSA	A	1:20	2	—
	BSA	A†	1:6·66	2	—
**	BSA	A	1:12·5	6	B 900305
**	pDLAla-BSA	A	1:5	10	B 900306
NSC 750	BSA	A	1:4	2·5	—

*Drug dissolved in ethanol solution.
†Drug dissolved in dimethyl formamide solution.
‡Complex formation in pH 8 phosphate buffer.
§Drug dissolved in dioxane solution.
‖Drug dissolved in dimethylacetamide solution.
¶Complex formation in pH 7 phosphate buffer.
**Ellipticine in the form of its hydrochloride salt.

identical for the complexes and the parent compounds. However, a number of the complexes show an increased degree of tumor inhibition;

(*b*) Only a few covalent conjugates (Method C, Table 1) were formed. In these cases, a behaviour similar to that reported for the nitrogen mustard derivatives [1] can be recognised, i.e., the optimal dose levels are shifted to higher doses;

(*c*) The effect of the various carrier proteins on the antitumor activity of a drug is different. The use of poly-alanyl proteins seems to offer a consistent advantage in most cases. A similar observation has been made with respect to nitrogen mustard combinations [1,2]. The various polylysine combinations appear to exert different effects, but the pattern is not identical for each drug. The carrier must be capable of binding the drug in a manner that is unaffected by body fluids during the transport process but is reversible in the target cell. A separate study is planned to evaluate the binding parameters of these complexes as

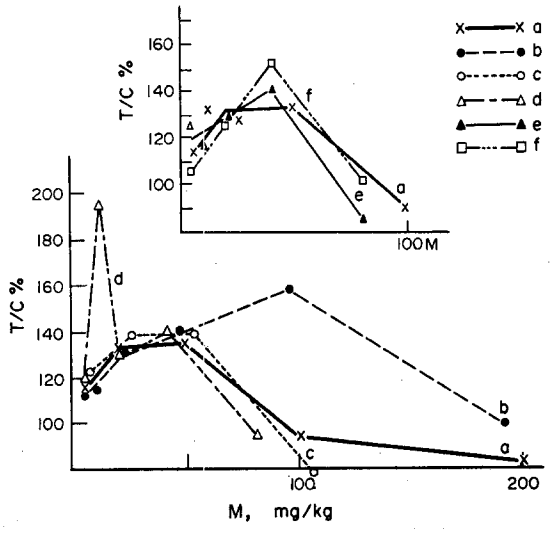

Fig. 2. *Effect of Actinomycin D* (*NSC* 3053)-*macromolecular combinations on the growth of the L* 1210 *lymphoid leukemia in BDF*$_1$ *mice; activity correlated to actual drug content.* (*a*) *NSC* 3053 (*B* 900325) (*b*) *NSC* 3053-*BSA, conjugate* (*B* 900326) (*c*) *NSC* 3053-*BSA, complex* (*B* 900327) (*d*) *NSC* 3053-*pDLAla-BSA, complex* (*B* 900328) (*e*) *NSC* 3053-*pDLAla—pLys, complex* (*B* 900329) (*f*) *NSC* 3053-*pGlu-pDLAla—pLys, complex* (*B* 900330).

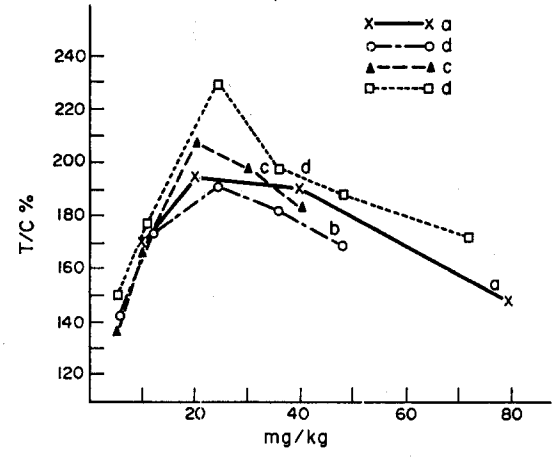

Fig. 3. *Effect of cytosine arabinoside* (*NSC* 63878)-*macromolecular combinations on the growth of the L* 1210 *system in CDF*$_1$ *mice; activity correlated to actual drug content.* (*a*) *NSC* 63878 (*B* 900331) (*b*) *NSC* 63878-*BSA, complex* (*B* 900333) (*c*) *NSC* 63878-*BSA, complex* (*B* 900340) (*d*) *NSC* 63878-*pDLAla—Fibrinogen, complex* (*B* 900334).

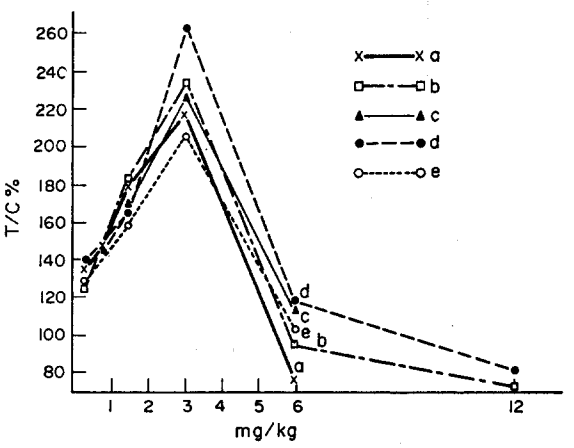

Fig. 4. *Effect of* 5-*azacytidine* (*NSC* 120816)-*macromolecular combinations on the growth of the L* 1210 *system in BDF*$_1$ *mice; activity correlated to actual drug content.* (*a*) *NSC* 102816 (*B* 900324) (*b*) *NSC* 102816-*BSA, complex* (*B* 900320) (*c*) *NSC* 102816-*pDLAla—BSA, complex* (*B* 900321) (*d*) *NSC* 102816-*pDLAla—Fibrinogen, complex* (*B* 900322) (*e*) *NSC* 102816-*pDLAla—pLys, complex* (*B* 900323).

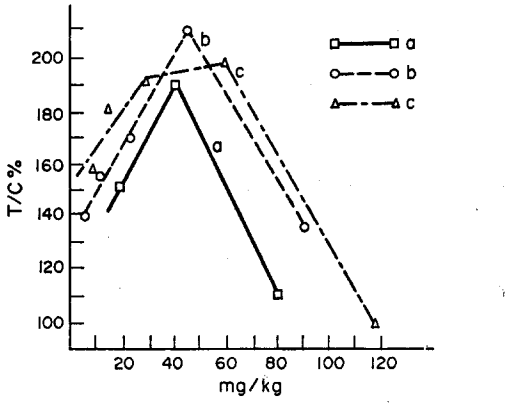

Fig. 5. *Effect of dichloromethotrexate* (*NSC* 29630)-*macromolecular combinations on the growth of the L* 1210 *system in BDF*$_1$ *mice; activity correlated to actual drug content.* (*a*) *NSC* 29630 (*B* 900317) (*b*) *NSC* 29630-*BSA, complex* (*B* 900318) (*c*) *NSC* 29630-*BSA, complex B* 900319).

well as the pH and temperature dependence on complex stability.

(*d*) In the case of Actinomycin D (Fig. 2), there is an indication that complex formation can result in reduced toxicity and an increase in the therapeutic index. If therapeutic index (TI) is defined as [the highest dose producing a T/C of 125% divided by the lowest dose producing the same response], then the parent compound, Actinomycin D (Fig. 2, B900325) has a TI of 4·3 while the Actinomycin D-pDLAla-BSA complex (Fig. 2, B900328) has a TI of 10.8. The BSA conjugate (B900326) and complex (B900327) of Actinomycin D are also superior giving TI values of 7·3 and 6·5, respectively. Further experiments are needed to evaluate whether this aspect is general. It appears that in favorable cases, a pharmacological effect can be obtained at distinctly lower drug concentrations. Such derivatives might possibly allow the more extensive use of a number of very active drugs that are presently used with difficulty due to their excessive toxicity.

Besides a decrease in toxicity, other advantages might be expected from macromolecular complexation. It has been reported [20] that the presence of macromolecules has a pro-

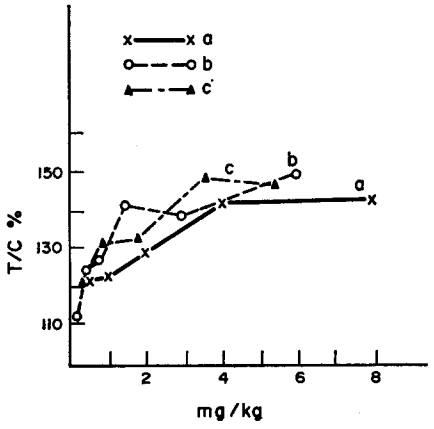

Fig. 6. Effect of NSC 141549-macromolecular combinations on the growth of the L 1210 system in CDF_1 mice; activity correlated to actual drug content. (a) NSC 141549 (B 900314) (b) NSC 141549-BSA, complex (B 900311) (c) NSC 141549-pDLAla—BSA, complex (B 900313).

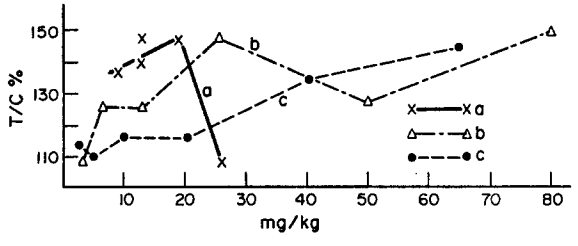

Fig. 7. Effect of cytembena (NSC 104801)-macromolecular combinations on the growth of the P388 lymphocytic leukemia in BDF_1 mice; activity correlated to actual drug content. (a) NSC 104801 (B 900303) (b) NSC 104801-BSA, conjugate (B 900302) (c) NSC 104801-BSA, complex (B 900304).

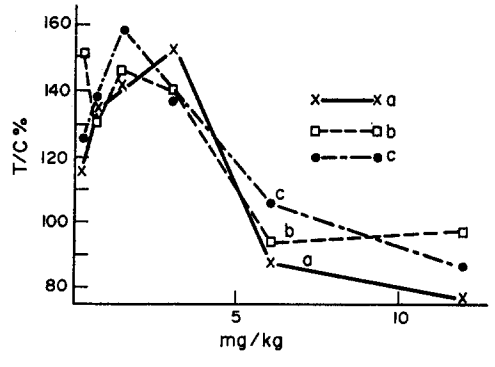

Fig. 8. Effect of NSC 163501-macromolecular combinations on the growth of the L 1210 system in BDF_1 mice; activity correlated to actual drug content. (a) NSC 163501 (B 900310) (b) NSC 163501-BSA, complex (B 900308) (c) NSC 163501-pDLAla—Fibrinogen complex (B 900309).

tective effect and slows down the rate of hydrolysis of some types of antitumor active nitrogen mustards in aqueous solution. This suggested studies with 5-azacytidine, an antitumor agent given by continuous infusion but which is rapidly decomposed in aqueous solution [21].

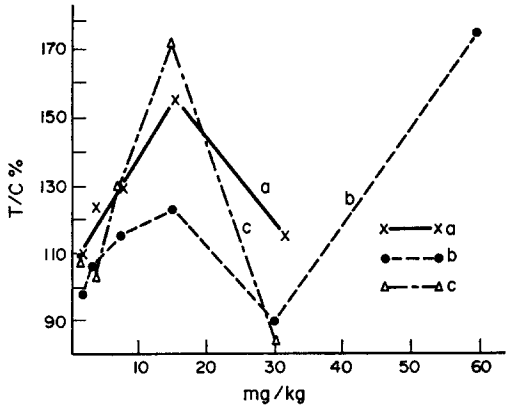

Fig. 9. Effect of ellipticine (NSC 71795)-macromolecular combinations on the growth of the L 1210 system in BDF_1 mice; activity correlated to actual drug content. (a) NSC 71795.(HCl) (B 900307) (b) NSC 71795-BSA, complex (B 900308) (c) NSC 71795-pDLAla—BSA, complex (B 900309).

Another possible advantage of complexation is related to the pharmaceutical formulation of water insoluble compounds. Complexation of these materials with water soluble proteins might enhance the solubility of the parent compound. An active, but relatively insoluble acridine derivative, NSC 141549 (Fig. 1) was used for this purpose.

The effect of complexation on the decomposition of 5-azacytidine was followed with u.v. spectroscopy. The change in u.v. absorption of 5-azacytidine at 243 nm was measured in the presence of different proteins in pH 7·3 phosphate buffer for periods of 30 min to 75 hr after incubation of 4° using a drug-protein ratio 1:9. Retardation of decomposition was largest in the first two hours (up to 30–40%) but with some of the carriers an effect could be demonstrated even 5 hours after incubation. These preliminary results are somewhat difficult to interpret since the binding characteristics of the decomposition products and the proteins are not yet available.

Protein complexes containing 5–9% of the acridine derivative (NSC 141549) retained activity and had better water solubility characteristics than the parent drug. The improvement, however, was not great enough in this instance to provide parenteral formulation possibilities.

DISCUSSION

A reason for an improved therapeutic efficacy with the use of macromolecular carriers may be related to the findings that the administration of radioactive albumin or fibrinogen leads to a relatively high incorpor-

ation of radioactivity into tumors [22–24]. De Duve suggested [9] the term "lysosomotropism" for drug-macromolecular combinations taken up by cellular endocytosis. According to this hypothesis the carrier is transported into the cell and there it is digested by lysosomal enzymes and the drug is released in its active form. The daunorubicin–DNA and adriamycin–DNA complexes were prepared based on this principle. Encouraging animal screening results [25, 26] resulted in clinical trials with these materials in the treatment of leukemia [25, 27]. Lysosomotropism was shown to be operative for the adriamycin–DNA complex [9].

A lysosome-mediated effect should be considered for drug–protein complexes. However, other mechanisms are also possible. Rubens and Dulbecco [28] have shown that chlorambucil and a tumor specific globulin are synergistic, but there is no requirement for physical absorption of the two materials since separate addition of the two components can still bring about the observed effect. The effect of simultaneous injection of separate solutions of the protein carriers and antitumor drugs described here is in progress. This will assess whether a similar synergistic action occurs with these materials. It is hoped that the investigation of complex stability and the nature of the bonds formed between the protein and cytotoxic drugs might offer important information for structure–activity relationships. There is certainly a need for further research work to establish the actual mechanism of antitumor action of the macromolecular derivatives.

With the development of tumor immunology, many investigators have sought to use antibodies to antigenic determinants expressed preferentially on tumor cells as carriers of cytotoxic agents in the hope of increasing the specificity of the drug and reducing its systemic toxicity. Several reports have appeared in which drug–antibody complexes [28–33] and conjugates, i.e., drugs covalently linked to immunoglobulins [3–5, 34] have been studied. For this approach to succeed, both the antibody and the toxic agent must retain activity when the two are linked together. The most powerful antitumor properties were achieved by first loading a poly-L-glutamic acid chain with the drug, followed by coupling of some of the chains to immunoglobulin [34]. This method also seems applicable in other cases, e.g., when hormone activity is to be saved, or when the covalent coupling reaction requires a chemical technique which would extensively denature the protein. We are presently involved in trying in such cases the application of the concept of the "inert intermediate carrier".

Although the idea of using tumor-specific antibodies as carriers is attractive, the isolation and purification of tumor-specific antibodies is presently still rather tedious, preventing general access to larger amounts of carriers of this type. We therefore feel that the exploration of readily obtainable non-specific macromolecular carriers might lead to combinations more easily exploitable for practical application.

An augmenting effect of macromolecules on cytotoxic drug action has been demonstrated and is supported by a number of experimental examples. It is hoped that the results obtained in these preliminary attempts to apply the principle of macromolecular carriers to antitumor drugs will stimulate further efforts in this area.

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Biological Activities of Murine Mammary Tumour Virus *In Vitro*

Increased Macromolcular Synetheses in Mouse and Hamster Kidney Cells; Production of B- and C-Particles in the Mouse Cells

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Abstract—*Murine mammary tumour virus (MTV) induced an increased net synthesis of DNA, RNA and protein in* short-*term cultures of baby mouse kidney cells (BMKC) made from two different BALB/c mouse substrains (He.A and Crgl.A). The dose–response ratio went through a maximum but growth inhibition was not observed. Similar effects were induced in baby hamster kidney cells (BHK 21/C 13).*

In longer-*term cultures of MTV-infected BMKC foci of piled-up small rounded epithelioid cells appeared on the monolayers. Cell lines derived from these foci grew well in media without serum and produced biologically active MTV (B-particles) and murine leukemia virus (MLV; C-particles). Higher* in vitro *passages of these cell lines induced less early (< 300 days) mammary tumours and more cases of leukemia than the lower passages after intraperitoneal injection. Cell-free extracts induced similar tumours. Not-infected control BMKC did not acquire the described properties. The nature of the induced MTV and MLV is discussed.*

INTRODUCTION

THE MURINE mammary tumour virus (MTV) has a B-type morphology and matures mainly in normal and oncologically transformed murine mammary gland epithelial cells [1–4].

As in human adults about 90% (in children less) of cancers are of epithelioid origin, MTV seems one of the most appropriate models for oncogenic viruses in man. Up to the present MTV can only be titrated with prolonged and large-scale *in vivo* assays [5–7]. So it is important to try to secure an *in vitro* assay for infectious MTV.

A few years ago the growth accelerating effect of highly purified MTV (B-particles) in *short*-term monolayer cultures of baby mouse kidney cells (BMKC) was found [8].

In the present communication we present results on MTV induced stimulation of macromolecular syntheses in *short*-term cultures of BMKC and BHK 21 cells. Further we report the morphological transformation of BMKC by MTV with concomitant induction of MTV (B-particles) release and of Murine Leukemia Virus (MuLV; C-particles) release in *longer*-term cultures. Parts of this work have been published in abstract form [9, 10].

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MATERIAL AND METHODS

Mice

BALB/c substrains He.A and Crgl.A were used for the preparation of BMKC cultures *in vitro*. Generally BALB/c mice get spontaneous mammary tumours at a rather high age (> 300 days) [11]. Substrain BALB/c/He.A does not contain B-particles in these late mammary tumours. But the mammary glands of old female animals were shown to contain MTV antigens and MTV biological activity [12]. Nevertheless this substrain can be used for the *in vitro* titration of MTV(S) (= standard

MTV) if care is taken only to count the early (< 300 days) mammary tumours [6]. BALB/c mice also carry endogenous mouse leukemia virus (MLV) [13, 14].

The endogenous MuLV manifests itself in BALB/c/He.A at a rather high age (Table 1): 75–80% of the leukaemia cases are generalized reticulum cell sarcomas, 15% are lymphoid and 5–10% are myeloid. Substrain BALB/c/Crgl.A, kindly given to us by Drs. K. B. De Ome and S. Nandi (Berkeley, California) is generally considered to be "free of MTV and MLV" [3, 15, 16].

Table 1. *Cation preferences for RDDP activities in media of A5 and A5* sub cell lines*

	Incorporation (counts/min) of dGTP-^{3}H in presence of		
Cells*	Mg^{2+}	Mn^{2+}	Mg^{2+} : Mn^{2+}†
A5	1853	2934	1:1·6
A5*	2478	30	1:0·01

*24 hr media of nearly confluent monolayers were used.
†Ratio of counts/min of dGTP-^{3}H incorporation.

Mouse cells

BMKC were prepared from 5 to 7-day-old male and female BALB/c mice and tissue cultured as essentially described previously [8]. Pimaricin 5 μg/ml was added to the medium. The calf serum was heat inactivated. The primary cultures were started with $5 \cdot 10^6$ living (trypan blue exclusion test) cells per Petri dish. On the 4th day the primary cultures (uninfected and infected; ±95% confluency) were detached by pronase (0·04% in Hanks' buffer) treatment for 3 min at 37°C.

Subcultures were prepared: one primary culture giving three secondary cultures. The medium was kept the same in all passages and was changed 3 times weekly. The secondary cultures consisted for 70–80% of epithelioid cells. The rest was fibroblastic.

Hamster cells

The BHK 21/C 13 cell line used is a cloned diploid Syrian hamster fibroblast line [17]. The cells were cultured in the same medium as the BMKC.

All cell types could be stored in liquid nitrogen [18].

Virus

Highly purified MTV(S) (standard MTV) was isolated from early (< 300 days) mammary tumours of BALB/c/He.A.f C3H.A [6, 19].

Infection of the cell cultures

Primary cultures were washed twice with Hanks' buffer solution on the third day and infected with 0·4 ml virus dilution in buffer solution containing 2% heat inactivated calf serum for 2 hr in the CO_2-incubator.

Cell-free extracts from morphologically transformed BMKC and mammary tumours

Transformed BMKC (100×10^6: ±0·7 g wet weight) were washed and homogenized with T.S.E. buffer (0·05 M Tris–HCl, 0·20 M sucrose, 0·001 M EDTA, pH 7·2) in a Potter–Elvehjem homogenizer (5 ml) during 1 min at 0°C.

Or 3 g of frozen tumours was thawed and cut into small pieces (±1 mm^3). Twenty-one ml TSE buffer, containing hyaluronidase (1 mg/100 ml) was added. After homogenization in a Sorvall Ommimixer (19,000 rev/min for 1 min at 0°C) the suspension was incubated at 23°C for 20 min. Every 5 min the precipitate was resuspended for half a min in the mixer. The homogenate of transformed BMKC or mammary tumours was centrifuged twice at 3300 *g* for 20 min at 0°C. Next the suspension was centrifuged at 10,000 *g* for 15 min three times. Pellets and floating material were discarded.

Finally a small transparant virus containing high speed pellet (Spinco L2, Rotor 50, 198,000 *g* for 15 min at 0°C) was obtained, which was resuspended in Hanks' buffer solution with the homogenizer and immediately used in the bioassay.

Electron microscopy and immuno-electron microscopy

The techniques are described elsewhere [20].

Biological tests

Three- to 4-week old female BALB/c mice were injected intraperitoneally with 10^6 syngeneic cells or an equivalent cell-free extract in 0·25 ml Hanks' balanced salt solution (BSS) and force bred [6].

Mammary tumours were classified either as early tumours (< 300 days) or as late tumours (> 300 days).

Determination of RNA-dependent DNA polymerase (RDDP) activity

Particle bound enzyme activities were measured in the high speed pellets of used media [21] with poly (rC). d(pG) 12–18 (Collaborative Research, Waltham, Mass.) and 8 mM $MgCl_2$ or 0·3 mM $MnCl_2$ as activator [22]. As a non-ionic detergent 0·1% (v/v) Nonidet P-40 (Shell) was used.

Determination of DNA, RNA and protein

The cells of each separate Petri dish were collected after trypsinization (0·25% trypsin in an isotonic saline sodium citrate (SSC), buffer, pH 7·0) at 37°C. The trypsinized cells were washed twice with ice-cold buffer, suspended in 1 ml ice-cold H_20 and if necessary stored at −28°C.

Subsequently the nucleic acids were extracted with $HClO_4$ [23] and quantitated [24, 25]. Purified [26] calf thymus DNA (BDH, Poole, England) and high molecular weight [27] yeast RNA (Sigma) were used as standards. Protein was determined in the acid insoluble fraction [28] with egg albumin (Sigma; 5 × cristallized) as a standard.

RESULTS

Stimulation of macromolecular syntheses in short-term cultures of BMKC by MTV

Many times, but not always, a maximum stimulation was found at virus dilution 10^{-3} or 10^{-2} (Table 2). A MTV induced inhibition was never found. Stimulated BMKC did not show cytopatogenic effects or loss of contact inhibition (no piling up of cells). Nor did they induce early mammary tumours in the bioassay, (not in the Table).

Stimulation of short-term cultures of baby hamster kidney cells by MTV

MTV appeared to induce similar stimulations in cultures of BHK 21/C 13 cells (Table 2). The percentages of stimulation are approximately the same for DNA, RNA and protein. Further, the Table shows that the number of cells per culture at the start of the experiment is critical. A too high cell density of the non-confluent monolayer at the beginning can mask the growth increasing effect by a more rapidly achieved confluency.

Table 2. Macromolecular contents of MTV infected kidney cell cultures derived from BALB/c/He.A mouse and Syrian hamster

Animal	MTV Batch	MTV Conc.	No.	DNA	RNA	Protein
Mouse	Mock	—	9	14.0 ± 4·3	7·5 ± 1·7	30·4 ± 2·9
	384	10^{-4}	6	16·6 ± 4·9	10·5 ± 1·7	44·8 ± 4·0
		10^{-3}	6	22·8 ± 7·5	15·3 ± 2·2	54·5 ± 8·1
		10^{-2}	6	19·9 ± 3·5	14·5 ± 1·7	62·2 ± 3·8
	385	10^{-4}	6	19·7 ± 4·3	12·8 ± 2·0	52·4 ± 9·0
		10^{-3}	6	26·4 ± 3·3	18·3 ± 4·2	70·0 ± 3·6
		10^{-2}	6	22·8 ± 7·8	16·3 ± 3·0	62·3 ± 6·2
Hamster	Mock*	—	6	78 ± 9·5	178 ± 17	404 ± 72
	351	10^{-3}	4	85 ± 7·7	239 ± 30	556 ± 22
		10^{-2}	4	114 ± 10·5	292 ± 12	629 ± 46
	Mock†	—	6	215 ± 18	451 ± 35	936 ± 46
	351	10^{-3}	4	236 ± 14	460 ± 15	964 ± 25
		10^{-2}	4	232 ± 11	448 ± 21	961 ± 30

DNA, RNA and protein expressed in μg/Petri dish.
The arithmetic means ± standard deviations are shown.
Mouse cells were harvested 4 days p.i. Hamster cells were grown in 10 cm diameter Petri dishes, infected with MTV in suspension at the start and harvested 5 days p.i.
*Started with $0{\cdot}9.10^6$ cells/culture.
†Started with $1.2.10^6$ cells/culture.

Morphological transformation of long-term cultures of BMKC by MTV

In longer-term monolayers of MTV(S) infected BALB/c-BMKC from both substrains foci of smaller rounded up cells appeared. These foci rapidly grew out to macroscopically visible 3-dimensional aggregates (pocks) of small round cells (Figs. 1–4).

We did not get spontaneous transformation in uninfected BALB/c-BMKC. Cell lines could easily be derived from the individual pocks. In contrast to the non-infected control BMKC the transformed cells grew equally well when the serum was omitted. With May–Grünwald–Giemsa staining the transformed BMKC showed a brilliant dark blue colour while the non-transformed cells showed a light-violet colour. Under our conditions the morphological transformation was a rather rare event while the preceding growth stimulation was a general phenomenon. In BALB/c/He.A BMKC the foci appeared once after 47 days (3 foci in 2/5 cultures infected with MTV dilution 10^{-3}) and once after 118–122 days (3 foci in 2/4 cultures infected with MTV dilution 10^{-3}). In BALB/c/Crgl.A BMKC the foci appeared after 18 days (6 foci in 3/3 cultures infected with MTV dilution 10^{-1}).

Release of MTV (B-particles) and MLV (C-particles) by MTV(S) transformed BMKC and their oncogenicity in vivo

The transformed BMKC produced and released both B- (Fig. 5) and C-particles (Fig. 6). When these cells were incubated with *rabbit anti-MTV* followed by incubation with ferritin-labeled goat antirabbit IgG, the B-particles were labeled (Fig. 5, 6) but the C-particles were always unlabeled (Fig. 6). The double oncogenicity of two such MTV(S) transformed BALB/c/He.A—BMKC cell lines (A4 and A5) is represented in Table 3. The A5 cells (passage 3) increased the natural low incidence of early mammary tumours in BALB/c/He.A mice and decreased the age at which these early mammary tumours could be detected. These mammary tumours contained B-particles (Fig. 7). Transformed A5 cells which had been cultured *in vitro* for a longer period (passages 8 and 9) increased the early mammary tumour incidence to a lower degree, but caused a considerable higher leukaemia incidence. Also with leukaemia a higher tumour incidence was correlated with an earlier manifestation of the tumours (Table 3). This corresponded with the electron microscopic observations. The number of B-particles went down and the number of C-particles even went up in the higher passages (Fig. 6). After passage 15 only a few B-particles could still be seen. Control BMKC were negative for B-, C- or budding particles, Only intracisternal A-particles were observed in the controls. A cell-free extract of A5 cells (8th and 9th passage) gave the same type of tumours as the whole cells (Table 3). Further the A5 cells could be inoculated subcutaneously with similar results (Table 3). The experiment with the A4 cell-line gave a result comparable with the outcome of the larger experiment with the A5 cell-line (Table 3). Early mammary tumours induced by A4 cells were used again

Table 3. Tumour incidences in ♀ BALB/c/He.A mice inoculated with MTV(S) morphologically transformed syngeneic BMKC (cell lines A4 and A5)

No. of mice	Cell line		Inoculation		Mammary tumours				Leukaemia	
					< 300 days		> 300 days			
	No.	Passage	Amount (cells)	Route	%	Age (days)	%	Age (days)	%	Age (days)
24	Mock	—	—	i.p.	13	281 ± 9	33	404 ± 59	21	380 ± 37
15	A5	3°	1×10^6	i.p.	73	205 ± 35	20	404 ± 29	7	363
10	A5	8°+9°	1×10^6	i.p.	30	218 ± 28	10	395	80	279 ± 110
9	A5	8°+9°	$0{\cdot}4 \times 10^6$	s.c.	22	208 ± 12	11	395	77	256 ± 60
11	A5	8°+9°	10×10^6	i.p. Cell-free	55	212 ± 23	18	367 ± 56	55	241 ± 62
7	A4	4°	1×10^6	i.p.	57	236 ± 42	43	403 ± 15	—	—

Cell-lines A4 and A5 were each derived from a single focus of morphologically transformed cells in two different experiments. The time of *in vitro* growth between the first detection of the transformation and harvesting for the bioassay was in the case of A4, fourth passage 43 days, in case of A5, third passage 39 days, A5 eighth passage 83 days and A5, ninth passage 98 days.
A5 cells of the eighth and ninth passage were pooled in the ratio 1:1.

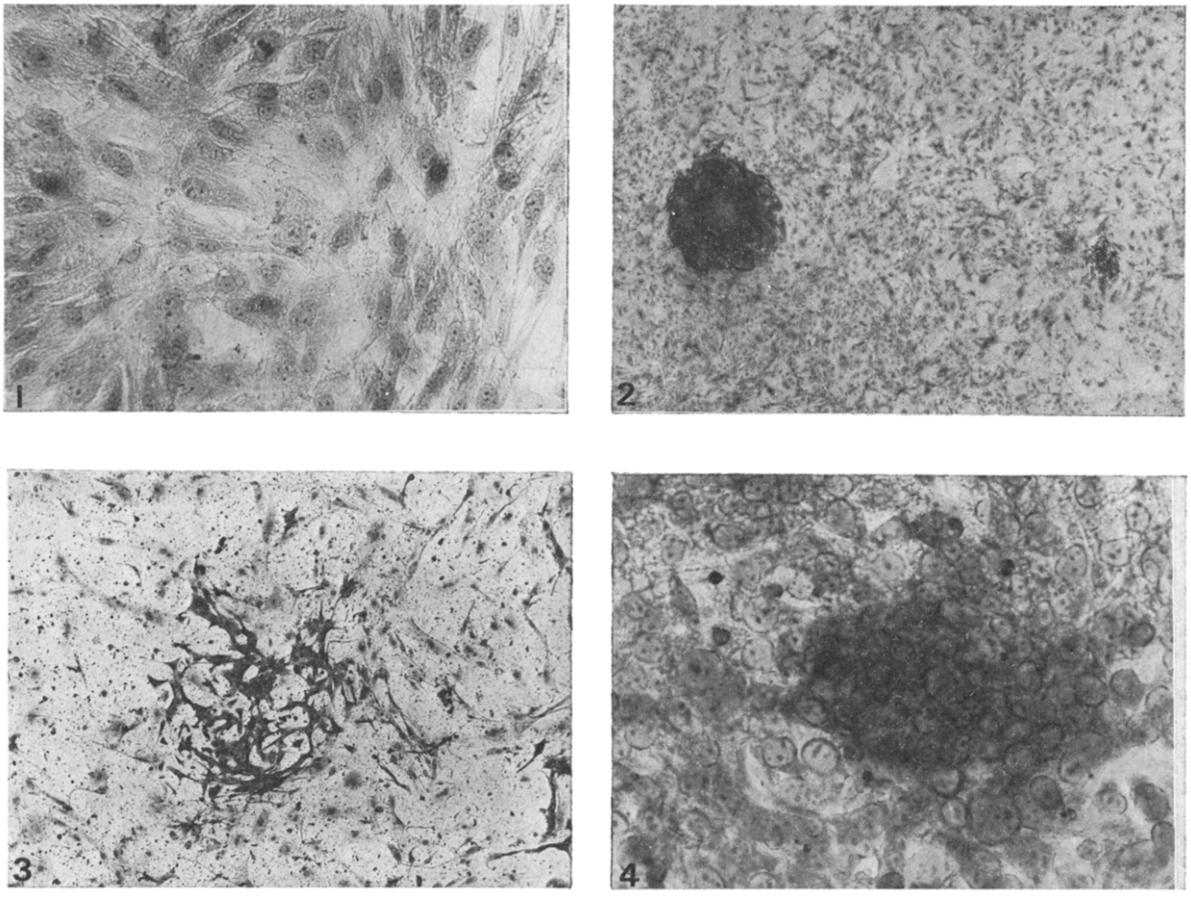

Fig. 1. *BALB/c/He.A—BMKC monolayer, second subculture,* 16 *days after starting primary culture.* × 180

Fig. 2. *MTV-induced focus of altered cells (pock) in BALB/c/He.A—BMKC monolayer.* × 9·5

Fig. 3. *As Fig.* 2*; less far developed pock.* × 38

Fig. 4. *Pock in culture derived from a single pock like the one in Fig.* 3*;* 11*th subculture of A*5*-cell line.* × 290

(*As Figs.* 1–4: *May–Grünwald–Giemsa staining*).

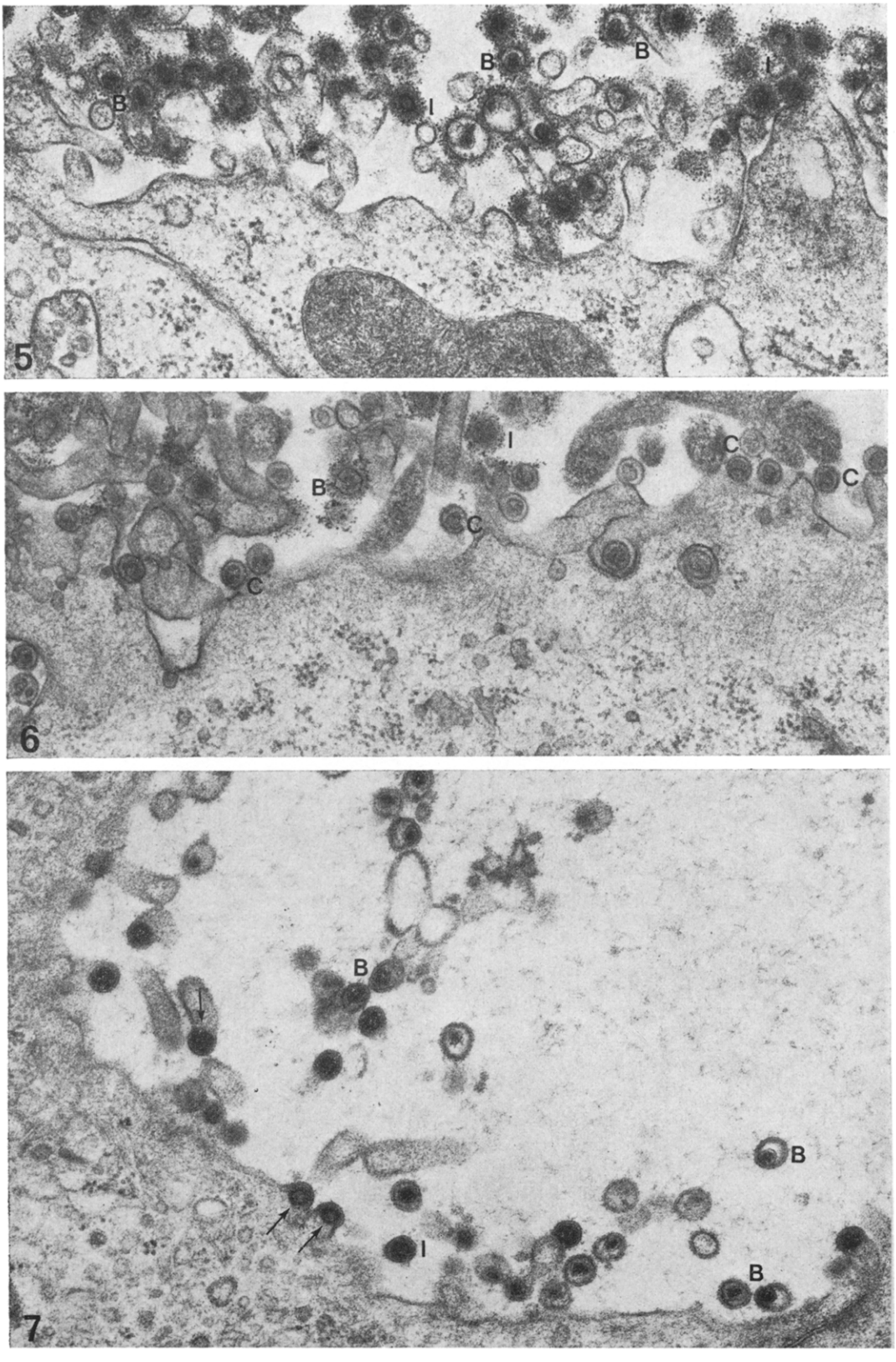

Fig. 5. *Transformed BMKC (cell line A5) after passage* 4, *incubated with* rabbit anti-MTV. *Mature (B) and immature (I). B particles are labeled.* × 48,000

Fig. 6. *Transformed BMKC (cell line A5) after passage* 15, *incubated with* rabbit anti-MTV. *Many C (C) particles, always unlabeled, and some mature (B) and immature (I) labeled B particles are present* × 47,500

Fig. 7. *Mammary tumour of BALB/c mice inoculated with transformed BMKC after passage* 3. *Mature (B) immature (I) and budding (arrows). B particles are present.* × 40,000

to prepare a cell-free extract. This extract once more induced early mammary tumours at an age of 182 ± 11 days in all five inoculated hosts (not in the table).

Heterogenicity of MTV(S) transformed BMKC

The A5 cell-line was derived from a single focus (pock) of morphologically transformed BALB/c./He.A—BMKC (Table 3; Methods). Viable A5 cells of the sixth passage (59 days after first subculturing the pock) were frozen and stored. A month later an ampoule with 10×10^6 A5 cells was thawed and distributed as equally as possible over two 10 cm diameter Falcon plastic Petri-dishes. Petri-dish No. 1 showed the expected growth pattern of an epithelioid monolayer with 3-dimensional aggregates (pocks) of rounded cells. Petri-dish No. 2 showed an epithelioid monolayer of more slowly growing cells, which did not form pocks. Subsequent sub-cultures from dish No. 1 and dish No. 2 maintained these different growth patterns. Determinations of the RNA-dependent DNA polymerase activities were carried out in the high speed pellets of media of both A5 cell lines. The ratio of the Mg^{2+} stimulated (largely specific for MTV) and Mn^{2+} stimulated (largely specific for MLV) activities was 1:1·6 for the A5 cells and 1:0·01 for the A5* cells (Table 1). The A5* cells apparently produced much less C-type virus per culture while the B-type virus production kept the same order of magnitude [22].

DISCUSSION

The increased net synthesis of DNA, RNA and protein in MTV infected BMKC, made from two different BALB/c substrains has been studied in more detail. A maximum stimulation was found at a rather high MTV(S) concentration. Still higher virus concentrations were less effective but never caused growth inhibition (Table 2). Some kind of auto-interference mechanism was apparently involved. It may have been caused by homologous incomplete MTV-particles which competed with non-defective MTV-particles at particular receptor sites [29] or our purified physically homogenous B-particles consisted of two (or more) competing types representing two (or more) MTV strains with different biological properties [4, 6, 19, 30–32]. The auto-interference did not act via interferon-like substances [33, 34].

Relevant to our experiments are the results of Lasfargues *et al.* [35] who reported that purified MTV stimulated growth and differentiation in epithelial and stromal elements of mouse mammary organ cultures.

Nair and De Ome [36] described a non-purified high molecular weight factor, which was released by cultured BALB/cfC3H. Crgl mammary tumour cells and which stimulated DNA synthesis in syngeneic BALB/c mouse embryo cells. We think this factor could have been closely related to the growth stimulating components of the MTV virion in our experiments.

Striking is the growth stimulating activity of purified MTV virions in baby hamster kidney cells (Table 2) [37]. The BHK 21/C13 cell line is fibroblastic but may give rise to epithelioid subclones [38].

Recently, similar early post infection increases of cellular DNA synthesis and occasionally of RNA synthesis (and cell multiplication) were reported for tissue cultured animal embryonic cells infected with unpurified C-type oncorna viruses [39–43].

Here the contamination could have caused mimicking effects [44].

It should be recalled that increased cellular DNA synthesis (and stimulation of mitosis) in tissue cultured cells are not only induced by oncorna viruses [45].

A second and rarer biological effect was the MTV induced morphological transformation in long-term BALB/c—BMKC monolayers. These morphologically transformed cells appeared to simultaneously produce MTV (B-particles) and MLV (C-particles) Figs. 5 and 6.

In connection with this *in vitro* two-step mechanism we recall that in most oncogenic process *in vivo* an early localized proliferation of non-neoplastic cells stands out prominently [15, 46].

All cell-lines derived from separate foci of transformed cells (pocks) grew rapidly out into epithelioid monolayers on which pocks were formed again. The comparable good growth in serum-less medium corresponds with their epithelioid nature [47].

The morphologically altered BMKC did not grow out into ascitic tumours after intraperitoneal inoculation (compare [48–50]), but induced early (< 300 days) mammary tumours and leukemia by means of released MTV and MLV (Table 3 and Results).

An important question is the nature of the released MTV variant. In the present case of MTV(S) infected BALB/c—BMKC one can surmise activated endogenous MTV but just as well the progency of the exogenously applied MTV(S). Also genotypic and phenotypic

hybrids of endogenous and exogenous MTV cannot be excluded. Endogenous MTV can be induced in BMKC from BALB/c mice, at least chemically, as we show in a following publication [57]. However it is not yet possible to distinguish the various MTV variants by immunological methods [31, 32], nor by any other good test. A similar question arises to a lesser degree with the released MLV.

Exogenous MLV can probably be excluded as similar highly purified B-particle preparations did not contain C-particles when controlled with the electron microscope [19] and showed no cross reaction with anti-Rauscher leukemia virus antisera in microimmunodiffusion tests [32]. Different endogenous MLV variants can be induced rather easily by various means as is well documented [21, 51, 52].

If we may assume that the released MLV is really of endogenous origin it is conceivable that the described induction of MLV by MTV could play a role in the spontaneous *in vivo* transformation of murine mammary adenocarcinoma into sarcoma [53–56].

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Immunological Depletion Contributing to Familial Hodgkin's Disease*

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Abstract—*The occurrence of Hodgkin's disease in three sisters of a sibship of five girls, over a period of six years, provided an ideal situation for the investigation of possible aetiological factors. Of note was the occurrence of recurrent herpes labialis in the fourth girl, infectious mononucleosus in the fifth sister and recent Herpes Zoster in the mother. The main findings were (a) a familial depletion of T lymphocyte function as evidenced initially in all members of the family by depressed lymphocyte transformation and (b) the presence of HL-A 1,8 in two affected sisters, the two unaffected sisters and the mother, who had, however, transmitted these antigens in the normal fashion. Of significance was consistent skin anergy to DNCB and tuberculin in the mother (HL-A 1,8) and in the fourth daughter (HL-A 1,8) even two and a half years after she had glandular fever. In addition, the mother, who has no outward signs of disease, had a paraprotein in her serum forming almost 60% of the gammaglobulin fraction.*

INTRODUCTION

FAMILIAL Hodgkin's disease is a well recognised entity, the risk to near relatives being almost three times that of the general population [1, 2]. In this family, where three sisters developed the disease (Fig. 1) we had an ideal opportunity to evaluate aetiological possibilities. The parents, the five sisters and the infant son of the third patient to present were studied. Studies undertaken included tests of T and B lymphocyte function, specific virus titres and genetic studies particularly chromosomal analysis and human leukocyte antigen patterns.

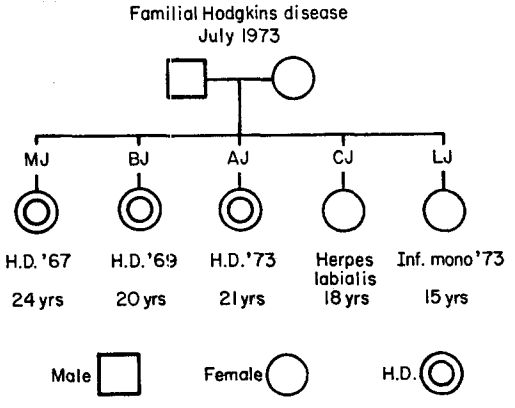

Fig. 1.

MATERIAL AND METHODS

M.J., age 18 years, (date of birth 6/8/'49) presented on 27/6/'67 with bilateral supraclavicular node enlargement and massive enlargement of paratracheal and hilar lymph glands on chest X-ray. A histological diagnosis of nodular sclerosing Hodgkin's disease was made. Initially, the patient received radiotherapy to the involved areas but later was commenced on the MVPP (nitrogen mustard, vinblastine, prednisone, procarbazine) chemotherapeutic regime because of infradiaphragmatic involvement, elevated ESR and anaemia. She has now been off all treatment since Spring 1975 and is in very good health.

B.J., age 16 years (date of birth 28/8/'53) admitted 16/12/'69 with a firm tender sternal mass, present for eight months, and a left supraclavicular node. Again histology revealed nodular sclerosing Hodgkin's disease and, following initial radiotherapy, she also required quadruple chemotherapy because of recurrent abdominal disease. She is now on therapy at 6 monthly intervals and is scheduled

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to stop the treatment. She also remains clinically in complete remission.

A.J., married, age 21 years (date of birth 6/10/'51). On 30/5/'73 presented, when six and a half months pregnant, with a history of increasing malaise for about six months. She had enlarged cervical and axillary glands and a swelling of the anterior chest wall. Node biopsy showed mixed cellular Hodgkin's disease. This patient had had an abortion in October 1972 at two and a half months pregnancy. Active therapy was withheld until the delivery of a healthy male infant on 3/7/'73, after which the patient was commenced on quadruple chemotherapy consisting of MVPP. However, E.S.R. was persistently raised on this regime and she was subsequently treated with varying combinations of epipodophyllotoxin, bleomycin, CCNU, vinblastine and adriamycin. At present she is asymptomatic, with normal E.S.R. Specific histological confirmation of diagnosis in the three girls is shown in Fig. 2.

L.J., age 15 years (date of birth 9/5/'57) developed infectious mononucleosus, April 1973, otherwise no illness of note.

C.J., age 18 years (date of birth 21/1/'54), history of recurrent herpes labialis, otherwise clinically normal.

Both parents are apparently healthy, with no clinical evidence of disease.

Family history

(Figure 3)—the maternal grandmother recalled two male sibling deaths at 4 and 7 months of age from? "viral infection". A 14-year-old sister of the mother had died following a brief illness in which she became lethargic and anorexic and about one month prior to her death developed a small crusted scalp lesion which had become increasingly large prior to her death. Unfortunately, the doctor who had attended this girl had died and the hospital record file has been destroyed by fire. A two month old male sibling of the mother had also died—"viral pneumonia". On the paternal side, deaths had occurred from natural causes and there was no history of early or inexplicable death.

Investigations

Immunological assessment—T cell function was assessed using the DNCB and PPD skin tests and *in vitro* lymphocyte transformation test using PHA as stimulant. A total lymphocyte count and serum immunoglobulin estimation was also done. Virological studies included EBV titers, herpes simplex and cytomegalovirus antibody titers. Genetic studies comprised detailed blood group analysis, chromosomal studies and leucocyte HL-A patterns.

RESULTS

The most striking finding was a familial immune depletion of T lymphocyte function (Table 1) as evidenced by consistently negative

Table 1. *Tuberculin and dinitrochlorobenzene skin tests in familial Hodgkin's disease (Family J) showing anergy not only in the three family members with Hodgkin's disease but also in the mother and L.J.*

	P.P.D.	D.N.C.B.
M.J.	–	–
B.J.	–	–
A.J.	–	–
L.J.	–	–
C.J.	+	+++
Mrs. J.	–	–
Mr. J.	+	+++

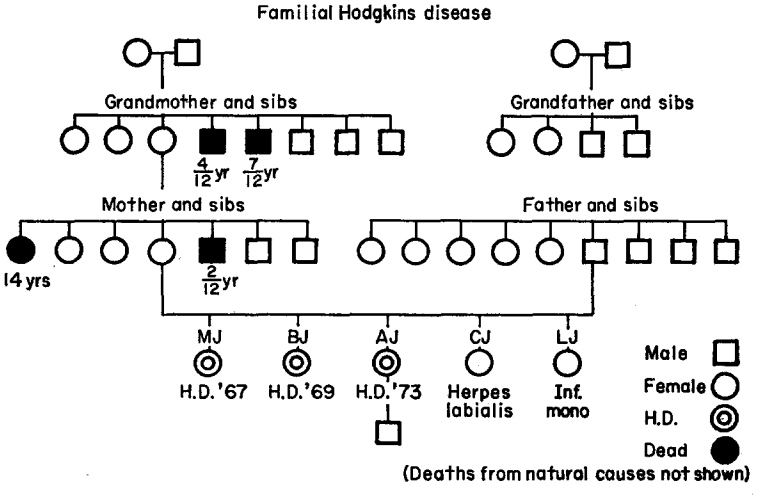

Fig. 3.

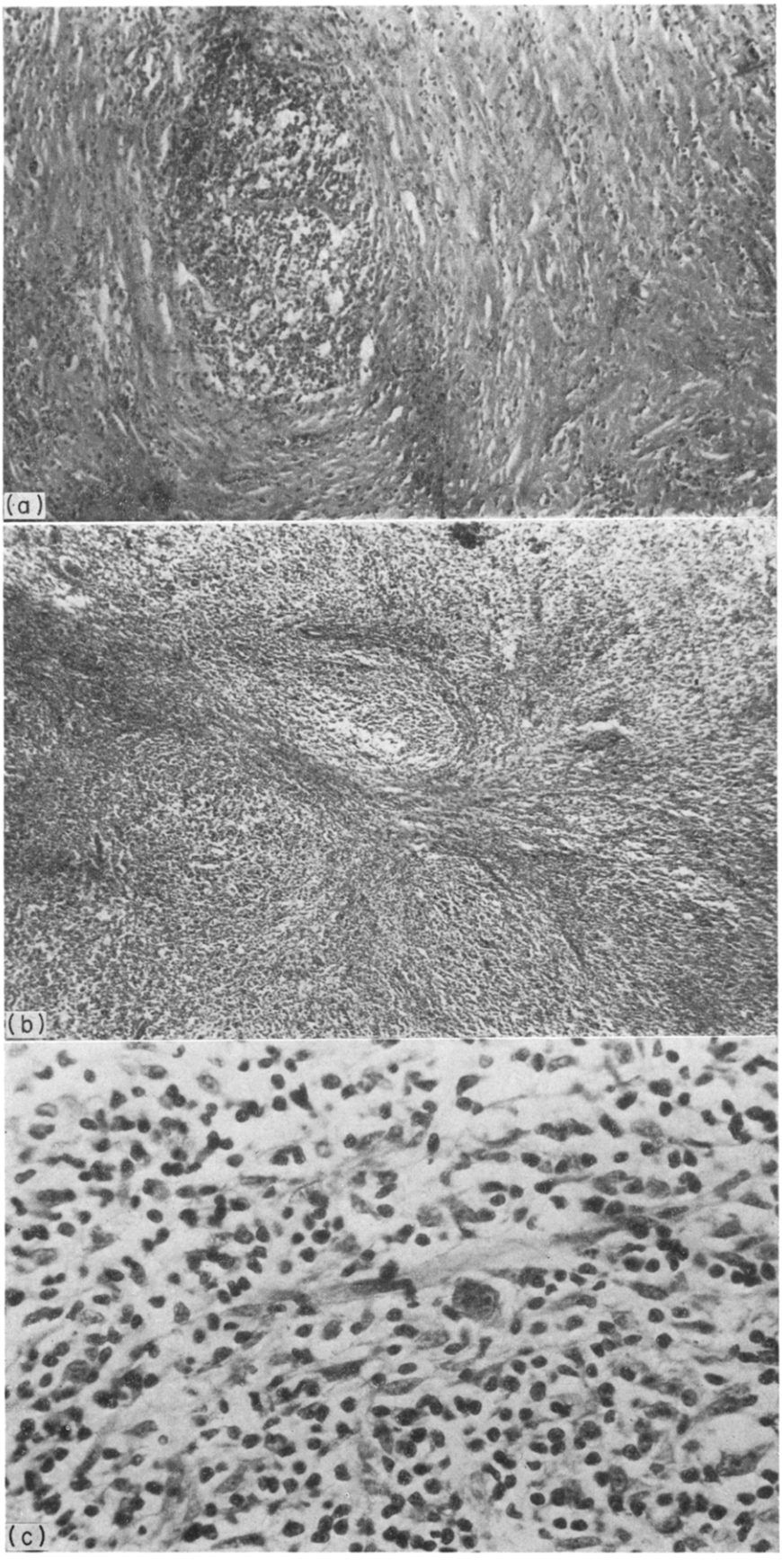

Fig. 2.

skin testing in L.J. who had infectious mononucleosus in March 1973, and a similar skin anergy in the mother on repeated tests. The three girls with Hodgkin's disease were anergic also and had poor lymphocyte blastogenesis, but they were on treatment at the time of study. Skin testing was normal in the father and in C.J. The *in vitro* lymphocyte transformation test (L.T.T.) was abnormally low in all the family members as compared to a normal control and the normal response of

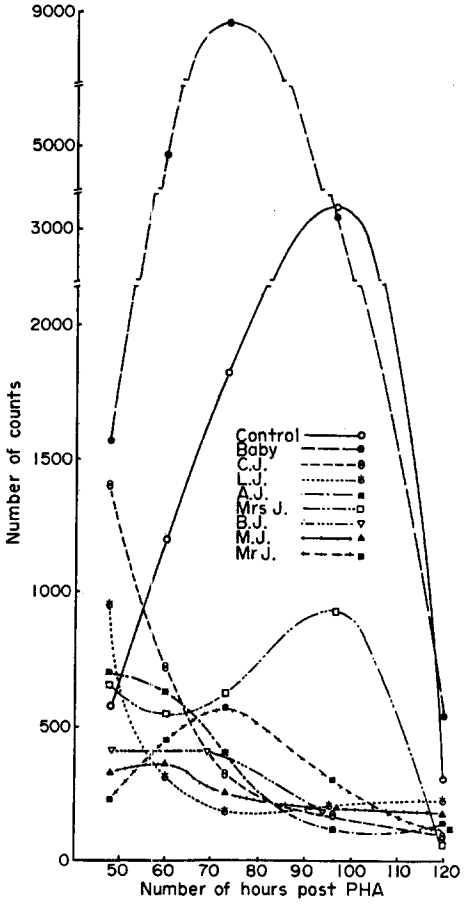

Fig. 4.

A.J.'s baby (Fig. 4). The result was considered very striking and was repeated within three months, again yielded a similar result. On reassessment in September 1975, however, the L.T.T. curve was now in the normal range, the mother and L.J. having a normal lymphocyte transformation, despite their persistent cutaneous anergy. The father and C.J. now showed normal *in vitro* blastogenesis, and their skin responses were, as before, normal. The mother was clinically normal until recent development of Herpes Zoster and there was no elevation of E.S.R. neither in her nor in the other unaffected members of the family. Virological studies i.e., E.B.V., Herpes Simple x Toxoplasma, in general, yielded insignificant antibody titers. Chromosomal studies were normal in all family members apart from aneuploidy in the three affected girls attributed to previous chemotherapy and radiotherapy. A detailed blood group analysis was normal. The result of the human leucocyte antigen system patterns are shown in Fig. 5. HL-A 1,8 were present in the mother, two of the three affected girls and the two unaffected girls. These antigens have been associated with Hodgkin's disease [3] particularly where there is an altered immunological response [4].

The total lymphocyte count in the family members was normal—lymphopenia in M.J. and B.J. was associated with treatment, but had been normal at initial presentation. Immunoglobulin studies revealed little apart from a depression of IgA in M.J. and the paraprotein in the mother.

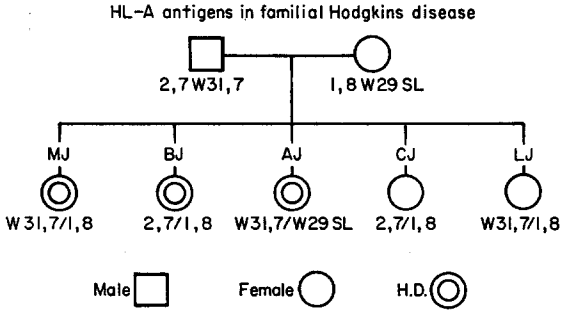

Fig. 5.

DISCUSSION

In this familial instance of Hodgkin's disease, a combination of an environmental factor and a genetic susceptibility or predisposition may have been the operative mechanism [5]. An added factor may have been a linkage between the HL-A genes and the genes controlling immune responsiveness [6]. In our study, we feel an immunological depletion of the thymus dependent "T" lymphocyte has been genetically transmitted from the mother to her daughters rendering their host defences low against an infective agent [7] possibly the Epstein–Barr virus [8–11] resulting in a transformation of the thymus dependent lymphocyte [12]. This infective process might then be presumed to have manifested itself as infectious mononucleosus and recurring herpetic infections in L.J. and C.J., as an asymptomatic paraproteinaemia in the mother, and as Hodgkin's disease in three members of the family. We are unable to explain the discrepancy or lack of

consistency between the latest lymphocyte transformation testing and the skin testing, however, the latter is strikingly negative in the mother and L.J. following repeated applications of D.N.C.B. and P.P.D. One would expect that the anergy associated with infectious mononucleosus should have transformed to allergy two and a half years later in the case of L.J. It would be difficult to discern whether the disease in this family may have been the result of person to person transmission or whether the inherited HL-A antigens 1 and 8 in immunologically incompetent persons could reflect an inherent susceptibility to the disease in the affected individuals [4, 13]. Of interest are the three early male deaths in the maternal pedigree and a very unusual death occurring in the mother's sister. One might speculate that in all four instances immunological depletion could well have been an important factor and ideally, one would wish to perform immune testing on the remaining members of the mother's family. The five girls in this sibship were the result of normal pregnancies and the mother had no history of abortion or stillbirth. The father runs a large hardware store and has been assisted by all members of the family in its management, there has been only rare contact with farm animals and the family keep no pets [13, 14]. Appendectomy and tonsillectomy [15] were considered to be of no aetiological significance, only one of the unaffected girls having had the latter. We feel a maternally transmitted immunological depletion or morphologic abnormality of the thymus derived lymphocyte, rendering it vulnerable to infection by an oncogenic virus or allied environmental agent with subsequent transformation and malignant proliferation, has occurred in the three patients here described with Hodgkin's disease.

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A New Metastasizing Mammary Carcinoma Model in Mice: Model Characteristics and Applications

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Abstract—*The criteria for a suitable experimental breast cancer model are discussed. Only one of a series of nine tumour systems initially studied, consistently showed early lymph node and late lung metastases and fulfilled the criteria. This mammary carcinoma* (2661) *was more fully characterized and was found to be non-antigenic following subcutaneous and intravenous immunity challenge tests. Size of the primary tumour as well as lymph node palpation were shown to be crude indexes for survival. Metastases formation gave rise to changes in lymphatic drainage pathways. Combination adjuvant chemotherapy increased the survival time in treated animals and produced a striking difference in patterns of metastases distribution. Results on immunity, surgery and chemotherapy in regard to this model are discussed.*

INTRODUCTION

IN THE experimental study of the formation of tumour metastases and the influence of surgery, radio- and chemotherapy it would be desirable to use an animal model which resembles the clinical tumour as much as possible [1–3]. Many of the reported breast cancer models consist of induced tumours which usually display marked antigenicity, in contrast with tumours of spontaneous origin [4–6]. Moreover, tumours which metastasize in a similar way as in man are rarely used.

The main and primary route of spread of mammary carcinoma in man is by way of the regional lymph nodes and the main cause of death is from haematogenous metastases. In mice, few tumours show both lymph node and blood borne metastases. While blood borne metastases are frequently observed [7, 8], lymph node metastases, if they occur, are seen only in a very low percentage [5, 9, 10]. Only a few model systems are known in mice which give lymphatic dissimination in a reproducible pattern but these only rarely produce parenchymatous secondaries. These systems use intratibial [11] intratesticular [12], tail [13], thigh and foot pad inoculation [14].

A suitable animal model for breast cancer should fulfil a number of criteria.

1. it should be transplantable and suitable for use in a syngeneic tumour host system and reproducible in this system.
2. the tumour should arise in the same organ for which it serves as a model and the antigenicity should be known and be low.
3. there should be a certain natural staging possible with regard to tumour spread.
4. the tumour should respond to the same therapeutic modalities as in man.

This paper describes the characteristics of a selected mammary carcinoma which fulfils the criteria mentioned.

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MATERIAL AND METHODS

Tumours

Several cell suspensions from tumour-lines of different histological types were injected subcutaneously into the foot pad. For each trial 2×10^5 viable tumour cells were inoculated in a volume of 0·02 ml with a Hamilton microsyringe. After local growth to a critical volume the tumours were removed surgically in order to prevent death from the primary. After death from residual disease all mice were autopsied

to record the extent of metastatic disease. Table 1 indicates those tumour–host systems initially studied. Only one of the tumours tested, mammary adenocarcinoma 2661, which originated spontaneously in a CBA/Rij mouse in 1961, gave metastatic growth in popliteal, inguinal, paraaortic and renal lymph nodes as well as lethal haematogenous metastases in the lungs. Therefore it was selected for further studies. Since a large number of cells of passage 61 had been stored in liquid nitrogen, the majority of the studies could be performed with passages 61–65. Tumour cell suspensions were prepared by the trypsinization method described by Reinhold [15].

Mice

All mice (inbred strains indicated in Table 1) were bred by brother sister mating in our own colony. For the experiments nine to ten week old CBA/Rij females weighing 20 ± 2 g, were the recipients for tumour transplantation. They were fed standard laboratory chow and tap water *ad libitum*.

Antigenicity studies

Two methods were employed to test the antigenicity of the selected tumour.

(*a*) End point dilution titration was carried out by a modification [16] of the method of Hewitt [17]. One further modification was used; the four injections per mouse were administered subcutaneously on the back of the animal, in the neck, the flanks and over the sacrum, rather than in the axillary and inguinal areas. This was done in order to facilitate differentiation between tumour growth at the inoculation sites and possible lymph node metastases. In order to test the suitability of the mammary carcinoma, tests were performed of the effect of prior immunization of the recipients.

(*b*) Boone *et al.* [18] provided a rapid intravenous assay technique of tumour immunity in mice as a substitute for the conventional procedure of Hewitt. Many studies have shown that most pulmonary tumour metastases in mice are found near or on the surface of the lungs [19–22]. Since the contrast between tumours and lung is adequate for counting after adding microspheres to the inoculate and after fixation of the lungs in Bouin's fluid, this method was employed instead of microscopically counting of tumour cell colonies from flattened lobes.

Evaluation of the presence of an oncogenic virus (mammary tumour virus) was done with the quantitative sepharose bead immunofluorescence assay with a polyvalent and a monospecific antiserum and purified mammary tumour virus antigens.

Surgery

Tumour removal was done under general anaesthesia by administering 60 mg/kg of body weight of sodium pentobarbital intraperitoneally, the line of amputation being between tumour and popliteal lymph node. The wound was closed with metal clips, which were removed ten days later.

Adjuvant chemotherapy studies

A combination of cyclophosphamide, 5-fluorouracil and methotrexate was used in a dosage used clinically by Bonadonna *et al.* [23]. This schedule was adapted from man to mouse on the basis of equal dose per m^2 body surface per 4 weeks [24]. The treatment was divided in four weekly cycles, so that each week the mice receive:

Cyclophosphamide*	100 mg/m^2 day 1, 2, 3	i.p.
Methotrexate*	20 mg/m^2 day 1	s.c.
5-Fluorouracil*	300 mg/m^2 day 1	i.v.

RESULTS

All the tumours tested gave rise to metastatic spread causing death after removal of the primary growth. All with exception of 1 line gave lung metastases (Table 1). One mammary adenocarcinoma (3641/75) showed a low percentage of lymph node metastases in addition to lung metastases. Mammary adenocarcinoma 2661 was the only one which gave early lymph node- and later lung metastases in 100% of recipients. Both lymph node and lung metastases can be easily detected at autopsy (Fig. 1). The typical morphology of the tumour cells allows recognition even at early stages of metastazation (Fig. 2, 3, 4).

Antigenicity studies of mammary adenocarcinoma 2661

(*a*) *Subcutaneous challenge.* It is well known that the presence of antigenic differences between tumour and host may interfere with tumour growth and tumour cell survival. To test the effect of prior immunization, two subcutaneous injections of 10^6 tumour cells that had been irradiated *in vitro* with 10,000 rad were given at two-week-intervals before injection of graded numbers of viable tumour cells. Titrations were then performed simultaneously in immunized and non-immunized control mice,

*Gifts of these drugs by Asta-Werke, Lederle and Hoffmann–La Roche respectively are gratefully acknowledged.

Fig. 1. *Animal killed* 40 *days after removal of the primary tumour. Metastases in inguinal- and axillary lymph nodes (popliteal and paraaortic lymph node metastases not exposed); as well as in the lungs (see insert).*

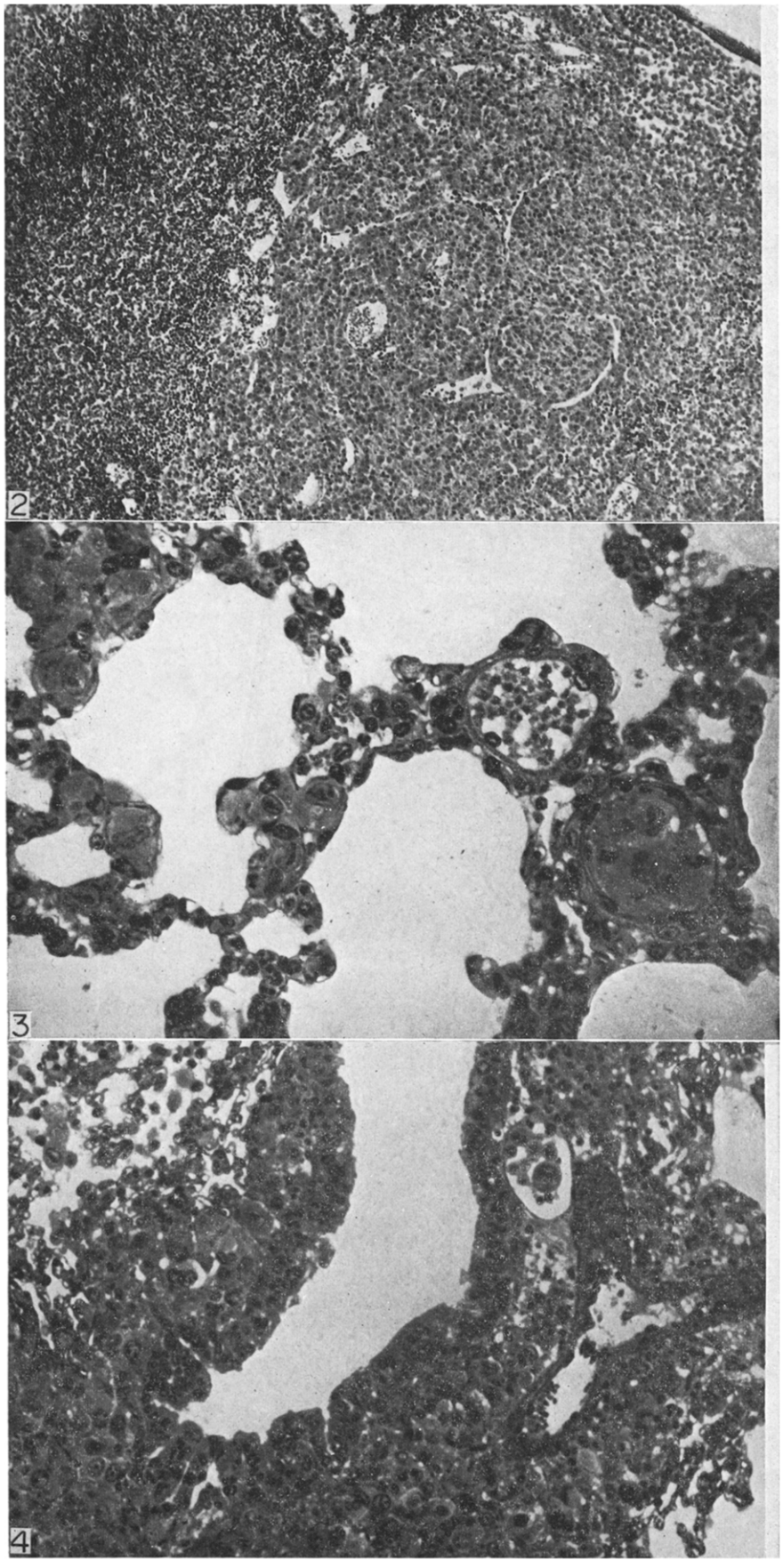

Fig. 2. Large metastase in lymph node. Methyl-methacrylate embedding, P.A.S. staining, original magnification 200 ×.

Fig. 3. Tumour cell emboli in capillaries and larger vessels. Methyl-methacrylate embedding, methenamine-silver staining. Original magnification 600 ×.

Fig. 4. Outgrowth of tumour cells around a bronchus. Methyl-methacrylate embedding, methenamine-silver staining. Original magnification 400 ×.

Table 1. *Tumour-host systems initially studied*

Tumour	Histology	Origin	Mouse strain	Lymph nodes	Lungs
18/76	adenocarcinoma	spontaneous	C57BLxC3H/f	—	+
3520/75	adenocarcinoma	spontaneous	C57BL/Rij	—	+
3641/75	adenocarcinoma	spontaneous	CBA/Rij	(10%)*	+
191/74	adenocarcinoma	spontaneous	C3H/f	−	+
2661/61	adenocarcinoma	spontaneous	CBA/Rij	+	+
30L/57	lymphoma	radiation-induced	C57BL/RijxCBA/Rij	+	−
C22LR	osteosarcoma	radiation-induced	C57BL/RijxCBA/Rij	−	+
3LL/51	sarcoma	spontaneous	C57BL/Ka	(10%)*	+
51CoL/76	carcinoma	chemically-induced	BALB/cxDBA/2	+	± (40%)

10 mice per trial.
*Regional lymph nodes only.

in both females and males. The results are presented in Table 2.

According to the Probit method [25] two curves were fitted to the two TD50 values per sex, the difference found was not significant ($P > 0{\cdot}10$). An analogous analysis by the Spearman–Kärber method gave similar information [26].

Table 2. *Effect of immunization on the TD*50 *(estimated number of cells necessary to obtain a tumour take in* 50 *percent of inoculation sites)*

Controls	Immunized
♀ 122	67
♂ 194	239

(*b*) *Intravenous challenge.* By intravenous injection of different numbers of tumour cells in the tail vein together with 10^6 microspheres the number of cells giving a significant and countable yield of metastases after 15 days was determined, as shown in Table 3.

Subsequently $2{\cdot}5 \times 10^5$ tumour cells were used for tumour immunity studies. Two groups of mice were inoculated intravenously at the same time. The first group consisted of mice from which a 10-day old tumour (10^6 cells in the flank) had been removed three days prior to challenge following the system of Boone *et al.* [18]. The average tumour volume was about 300 mm^3. The second group consisted of normal mice from which a skin flap had been removed, designed to mimic the trauma of tumour excision. three days prior to tumour challenge. Table 4 shows that there is no significant difference in the numbers of lung colonies developing in either group.

Table 3. *Relationship between the number of i.v. injected tumour cells and number of pulmonary metastases after* 15 *days*

Number of cells*	Average number of lung colonies ± S.E.M.
10^5	21 ± 4·9
$2{\cdot}5 \times 10^5$	41·6 ± 6·42
5×10^5	82·4 ± 19·0
$7{\cdot}5 \times 10^5$	innumerable
10^6	innumerable

*10^6 microspheres were added to each inoculum.

Table 4. *Number of lung colonies* 15 *days after i.v. inoculation of* $2{\cdot}5 \times 10^5$ *tumour cells into control and immunized mice*

		Average number of pulmonary metastases ± S.E.M.
Control	(15)	35·5 ± 3·289
Immunized	(15)	43·3 ± 4·211

($P > 0{\cdot}10$) Student's two-sample test.

The Sepharose bead immunofluorescence assay showed no antigens of the mouse mammary tumour virus [27].

Surgery and metastases formation

Complete cure in an observation period of 150 days can be obtained when amputation is performed before the 10th day whereas animals die from metastatic disease when amputation after 15 days is performed. In the experiments the median survival time of mice which die after removal of the primary tumour is 45 days. However, the spread of survival from different experiments is great, with several mice surviving over 60 days.

This range of survival in a homogenous system where standard tests did not reveal host resistance indicates the presence of random factors influencing the growth of metastases.

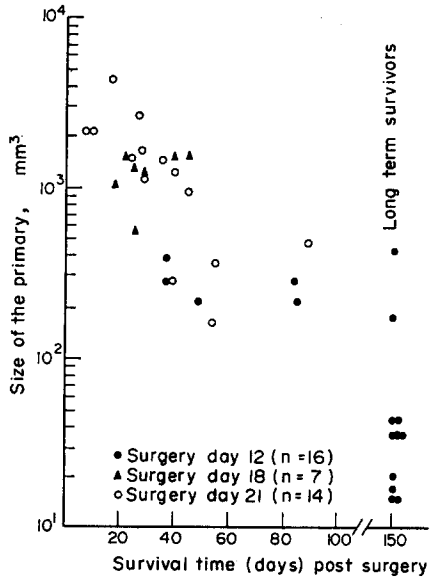

Fig. 5. Survival time post surgery as a function of the size of the primary growth.

Within the range of 10–15 days postoperatively there is no significant correlation between volume of the primary growth and the risk of metastases. Nevertheless mice that had tumours removed when less than 100 mm³ measured by 2 diameters at right angles and the thickness of the tumour, did not initiate metastases, whereas mice that had tumours removed of more than 500 mm³ all died of metastasic disease (Fig. 5). It therefore appears that, as with human breast cancer [28–30], size of the primary growth is a *crude* index of metastatic risk.

Lymph node metastases

The possibility of direct intralymphatic injection, as often used in lymph node metastases models, was unlikely in view of the absence of tumour spread if amputation is performed before day 10. In addition it was excluded histologically. Histological sections of the lymph nodes were made immediately after, 1 day after and every other day until 17 days after injection of the tumour cell suspension. Five female mice were used for each group examined, weights of lymph nodes were measured with a Mettler type B5-balance. The weight of the lymph nodes had already increased the first day after inoculation and continued until tumour cells appeared in the sinus* (Table 5). The first tumour cells appeared in the sinus of the popliteal lymph node

Table 5. Weight in mg of lymph nodes (±S.D.) draining mammary carcinoma 2661

Day	Popliteal	Inguinal	Paraaortic
0 (controls)	1·06 ± 0·09	2·80 ± 0·77	0·52 ± 0·18
1	1·82 ± 0·34	3·04 ± 0·26	0·76 ± 0·11
3	2·84 ± 0·44	3·24 ± 0·80	1·00 ± 0·21
5	3·46 ± 0·46	3·94 ± 0·35	1·46 ± 0·44
7	6·68 ± 1·57	4·64 ± 0·99	2·00 ± 0·90
9	8·04 ± 1·40	4·52 ± 1·05	1·68 ± 0·40
11	13·10 ± 2·15	6·36 ± 1·92	2·62 ± 1·14
13	10·82 ± 1·07	5·38 ± 0·43	3·20 ± 1·31
15	19·96 ± 0·93	9·92 ± 4·75	9·54 ± 3·45
17	25·48 ± 4·03	10·40 ± 0·95	9·76 ± 3·70

at day 13; at day 15 all popliteal lymph nodes contained tumour cells infiltrating and destroying the lymph node while other non regional lymph nodes showed the presence of tumour cells at this time as well. As shown in Table 5 the inguinal lymph node plays an active role as shown by enlargement and subsequent metastasis formation. This finding is surprising in view of the normal lymph drainage from the foot pad. When Patent Blue Violet (2·5% solution for lymphography) is injected into the foot pad in a way similar to tumour cell inoculation, the first node to stain is the popliteal (= regional) lymph node. Afterwards the paraaortic and renal lymph nodes are stained. Even when the liver is blue, indicating the presence of P.B.V. in the circulation, the inguinal lymph node remains unstained, suggesting that metastases formation itself produces changes in the lymphatic drainage pathways.

The nodal status of tumour bearing animals appears to be of interest: all animals have palpable popliteal nodes on day 12. This is mainly due to paracortical hyperplasia and increased germinal centre activity [31]. After surgical removal of the tumour on day 12 some of the nodes regress in volume, whereas others show an increase in volume due to metastatic growth. This phenomenon has also been observed clinically by Edwards *et al.* [32] after simple mastectomy. To investigate the prognostic value of the lymph node status in conjunction with the surgical removal of the tumour, animals were grafted with tumour cells into the foot pad. On day 12 after inoculation the tumours were removed and on day 20 all lymph nodes were palpated and the animals were divided into 3 groups as follows:

1. popliteal and inguinal nodes positive.
2. popliteal node positive and inguinal node negative.
3. popliteal and inguinal nodes negative.

*A detailed morphometric analysis of the immune response to an antigenic and this "non-antigenic" tumour is in preparation [31].

(Positive and negative as judged by enlargement on palpation.) Figure 6 shows the survival curves of these animals, together with untreated ones indicating that the nodal status is a reasonable prognostic indicator for survival.

Chemotherapy

Because of recent interest in the Bonadonna trial [23] we decided to test the drug combination used both for established tumours and in the postoperation adjuvant situation. For the former this was done utilizing objective response criteria (shrinkage of measurable tumour by greater than 50%, standard definition of objective regression). Flank tumours were used for practical reasons and therapy was started when tumours were well established in early log phase of measurable growth and was continued for 4 weeks. The results as shown in Fig. 7 indicate that this schedule is effective but if treatment is stopped all tumours resume growth; no toxic deaths occurred. Only when the dose was doubled mice died of severe bone marrow depression.

As adjuvant therapy this schedule was started 3 days following surgery. Surgical removal was done at 18 days after tumour injection into the foot pad, the average tumour volume being 1280 mm^3. In this stage some animals had already died of their primaries and lymph node metastases were already established. Figure 8 shows the survival of treated and untreated animals demonstrating a significant delay of survival in the treated group ($P < 0{\cdot}05$). A striking difference was found in the site of metastases in the treated group. Whereas all untreated mice showed large lymph node metastases in addition to lung metastases at autopsy; a significant reduction of lymph node metastases occurred in the treated group (Table 6) in contrast to their massive lung metastases. It is our impression that the lungs of animals in the treated group showed more heavy metastases than those from untreated mice dying at the same time.

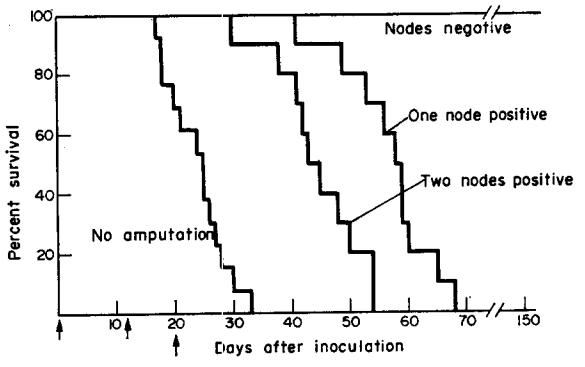

Fig. 6. Survival of mice dependent on node status and treatment. Positive and negative as judged by enlargement on palpation.

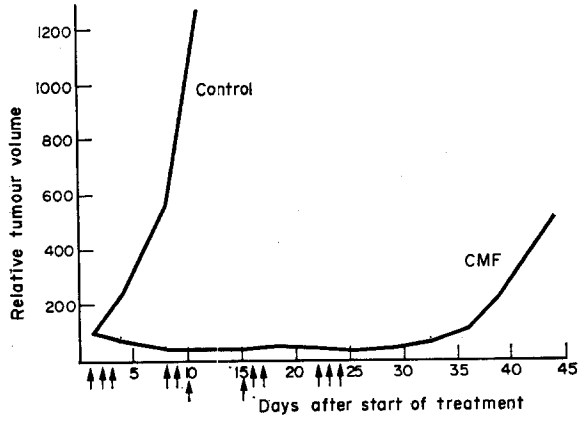

Fig. 7. Growth delay on flank tumours by combination chemotherapy.

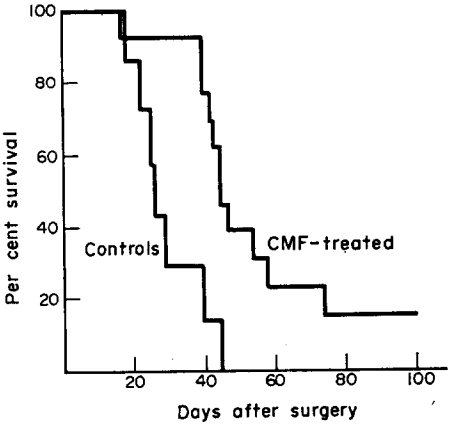

Fig. 8. Survival of mice after removal of the primary growth, given adjuvant chemotherapy.

Table 6. Reduction of lymph node metastases after adjuvant chemotherapy

	Percentage of positive nodes*	
Treated	22·7	$N = 11$
Untreated	100	$N = 6$

$P < 0{\cdot}001$, Cochran's test [49].

*Four nodal areas were examined. The value presented is the percentage of the total number of nodes positive/total nodes. The lymph nodes examined were the popliteal, inguinal, paraaortic and axillary on the ipsilateral side with respect to the primary tumour.

DISCUSSION

The limited insight currently available in the mechanisms of metastases formation may in part be due to limitations of experimental models used to approach this complex problem. With better models a rational basis to found attempts at influencing metastases formation may be investigated. This is especially true for cancers showing two modes of spread. Mammary carcinoma in man is one example: lymph node- and subsequent evidence of haematogenous spread of tumour occurs in general. The main finding which emerges from this work is that there is now an experimental model which shows these two modes of spread in 100% of cases. One way in which the model can be used is as a useful tool in testing effectivity of different types of therapy in different stages on metastases formation. However, we would stress that no animal tumour can represent all of the variations in growth potential and metastasizing characteristics that are encountered in humans. However, it is of significance in this system that the two modes of spread are both 100% phenomena, which may be unrealistic but very convenient to evaluate therapy differences. These special properties of metastases formation have not been met in other useful breast cancer models [1–3]. The validity of a therapeutic model of course should always be confirmed by findings of parallelism in response between the clinical tumour and experimental model.

Special attention was given to experiments testing whether the tumour is antigenic or not in its host of origin. Several investigators [33–35] found that antitumour immunity has a higher level of effectiveness in the lungs than in subcutaneous tissue. It is likely that the passage in the bloodstream for the former type of inoculum exposes the tumour cells more readily to the hazard of elimination by antibody; in the latter case cellular immune responses are more likely to be effective. For that reason an additional intravenous test according to Boone *et al.* [18], was added to the conventional one using subcutaneous challenge [17].

Both tests (see Tables 2 and 4) showed that no antigenicity could be detected, as one would expect for a syngeneically transplanted tumour of spontaneous origin in a low cancer strain. This in contrast to mammary tumour mouse strains such as C3H/+ having a vertically transmitted exogenous virus inducing the tumour. Spontaneous can be used in the true sense of the word here: No identifiable oncogenic agent was involved which could entail artefactual immunity.

The foot pad location was chosen intentionally: s.c. transplants of the mammary carcinoma in the flank of mice can grow to a very large size, yet metastasize with a low frequency. Inoculation into the foot pad leads to a tumour which metastasizes much earlier. Intramuscular inoculation gives lung metastases and a low percentage of lymphogenous metastases (unpublished results). This can possibly be explained by pure mechanical and anatomical reasons. There is an increased pressure in the foot pad rather than the flank when the tumour starts growing, promoted possibly by movements of the foot [36]. Muscle itself does not contain lymphatics. Lymphatics are however present in the fascial planes enclosing and dividing muscles.

The reason why so few rodent tumours metastasize via the lymph nodes is unknown. It is possible that intrinsic properties of the tumour cells themselves help to determine whether they enter local lymphatic vessels or are retained in the draining nodes. Similarly in man, it is unknown why it is common for carcinomas to metastasize to regional lymph nodes while most sarcomas do so infrequently; but differences in fine surface structure of the tumour cells may perhaps be implicated. There is no evidence that sarcoma cells are inherently less capable of invading lymphatic structures.

Whether there is any relation between a lack of immunogenicity and metastasizing capacity of tumours as stated by Davey *et al.* [37] and Kim *et al.* [38, 39] is uncertain. This is especially true because metastasizing tumour models are known with chemically induced antigenic tumours [3] from which the model of Carr *et al.* [40, 41] also utilises foot pad inoculation. Mammary carcinoma 2661 in its present state is an undifferentiated carcinoma. During transplantation passages most tumours change their histological pattern towards anaplasia; this tumour progression has been reported to go together with an increased tendency to form metastases of spontaneous as well as induced tumours by Rudenstam [42]. It is not known if this is the case here since the test for metastases was done only in its 61st passage but another mammary carcinoma (3641/75, Table 1) was tested in its first transplantation generation and also gave lung metastases in 100% of cases.

The ultimate reason why this particular murine tumour when inoculated via the foot pad gives rise to lymph node- and lung metastases remains unknown, as intralymphatic

injection was ruled out but this property makes this model a unique and valuable one. Time, tumour size and lymph node palpation were shown to be reasonable parameters for staging the disease. A late stage of the disease was used in chemotherapy experiments to examine whether cures could be obtained with chemotherapy against the primary tumour. Combinations of drugs have been shown to be more effective than single drugs [43, 44, 45]; for that reason the Bonadonna [23] schedule was applied without analysis of the sensitivity of the tumour to the single drugs. There were however no cures in either experimental groups (Fig. 7). When flank tumours were used, resumed growth occurred for all tumours after an objective regression. However they did not show an increased doubling time in the regrowth phase in contrast to the findings of Shewell [46]. When the same treatment schedule was used in a late stage adjuvant situation a striking difference was found between the sensitivity of lymph node and lung metastases not found in other models [3, 13, 14, 47, 48]. This should be evaluated in future experiments using single drugs and other combinations.

It is evident that this model which fulfils the criteria mentioned in the introduction has essential metastasizing and therapy response characteristics which permit evaluation of therapy combinations and therapy sequences. Although no animal model can completely mimmic mammary carcinoma in man, this model seems to be a very attractive experimental model in mice.

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Acquired Methotrexate Resistance in Lymphoblasts Resulting from Altered Kinetic Properties of Dihydrofoltate Reductase*

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Abstract—*We describe the properties of a dihydrofolate reductase purified from lymphoblastoid cells which acquired methotrexate resistance following long-term culture in presence of the drug. The enzyme showed marked differences in kinetic properties from the parental cell strain. The K_m for dihydrofolate was increased 18-fold, relative to the parent line, and the affinity of the enzyme for methotrexate was decreased by 50-fold. Binding of NADPH, however, was tighter than in the parent line, with a K_m of less than half the original value. The molecular weight of the mutant dihydrofolate reductase, as measured by gel filtration, was unaltered, but the mutant enzyme showed much greater heat lability than the wild-type enzyme. At low drug concentrations, cells possessing the altered reductase possessed a selective advantage over cells with normal dihydrofolate reductase. Increasing the selective pressure, however, by raising the methotrexate concentration in the medium above $1{\cdot}6 \times 10^{-6}$ M, led to selection against the mutant, and cells with normal type dihydrofolate reductase again dominated the population. These results are interpreted in the context of high-affinity and low-affinity sites of action of methotrexate.*

INTRODUCTION

THE CYTOTOXICITY of methotrexate (MTX) is generally attributed to its inhibition of the enzyme dihydrofolate reductase (EC 1.5.1.3) [1], although, in common with most antimetabolites, MTX inhibits at several metabolic sites. In human lymphoblastic cultured cells of the W1-L2 line these other sites of action include thymidylate synthetase (EC 2.1.1.b), serine hydroxymethyltransferase (EC 2.1.2.1) and the membrane transport of reduced folates [2–4]. However, binding of MTX at these secondary sites is 5 or more orders of magnitude weaker than the binding of MTX to dihydrofolate reductase [2, 3]. Nevertheless, the possible importance of these low affinity binding sites for MTX in determining therapeutic response to the drug remains a topic of current interest [5, 6].

Acquired resistance to methotrexate has been the subject of numerous studies, both *in vivo* and *in vitro* (reviewed in ref. 2). The two commonest mechanisms of resistance involve an increase in the cellular activity of dihydrofolate reductase, and a decrease in the permeability of the cell membrane to the drug. Other mechanisms may operate in particular cases; for example, in culture medium enriched with thymidine and a purine, most cells can continue normal growth in the presence of toxic MTX levels, by utilising the salvage pathways [7, 8]. Thymidylate synthetase activity may also be increased following exposure of cells to MTX [9, 10]. The different modes of resistance may occur in combination, and our previous study with W1-L2 cells [2] showed that the most resistant mutants obtained had

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Abbreviations used: MTX, methotrexate (amethopterin; ID_{50}, inhibitor concentration that gives 50% growth inhibition in 48 hr; IU, international unit of enzyme activity, = 1 μmole substrate converted/min.; E_t, total molar enzyme concentration; S, molar substrate concentration; I, molar inhibitor concentration; i, fractional inhibition

both impaired MTX transport and greatly elevated dihydrofolate reductase activity.

Several previous reports have examined the mechanism of the increase in dihydrofolate reductase activity, and the nature of the additional enzyme. In L1210 mouse leukaemia cells, moderately resistant mutants, showing a 7-fold increase in dihydrofolate reductase activity, were shown to synthesize enzyme molecules at 7 times the normal rate, and to degrade the enzyme at the normal rate; the enzyme had normal kinetic properties [11–13]. However, a highly resistant L1210 mutant, with the reductase increased 80-fold, only synthesized the enzyme at 65 times the normal rate, and it degraded the enzyme at one-third the normal rate [11]; this enzyme showed electrophoretic differences from normal L1210 dihydrofolate reductase, and the binding of NADPH was tighter than normal [13, 14]. Blumenthal and Greenberg, in a study with a MTX-resistant subline of mouse leukaemia L4946, showed that not only was total dihydrofolate reductase activity increased 20-fold, but that the enzyme in the resistant subline showed qualitative changes [15].

The kinetics of inhibition of dihydrofolate reductase are difficult to study accurately because of the quasi-stoichiometric nature of the inhibition by MTX. Some studies have indicated that the varying intrinsic sensitivity to MTX of different mammalian cell types may correlate with the tightness of binding of MTX by the cell's dihydrofolate reductase [16, 17]. The latter work showed that the Yoshida sarcoma was 70-fold more resistant to MTX than the L1210 leukaemia, despite the fact that the two cell lines had similar activities of dihydrofolate reductase, and similar rates of membrane transport of MTX; the reductase of the Yoshida cells, however, bound MTX 25-times less tightly than the L1210 reductase, and the Yoshida enzyme was thus much less readily inhibited [17]. Acquired resistance to MTX associated with loss of binding power for MTX has not hitherto been described. In the present paper we describe studies with two highly MTX-resistant sublines of the W1-L2 human lymphoblastoid cell culture line, one of which contained a dihydrofolate reductase with greatly altered kinetic properties.

MATERIAL AND METHODS

Chemicals

Methotrexate was obtained from Lederle Laboratories, Pearl River, N.Y. It contained less than 2% of folic acid and other contaminants, and was used without further purification. NADPH and dihydrofolate were purchased from Sigma Chemical Co., St. Louis, MO. The dihydrofolate was 92% pure, and was not purified further. AH-Sepharose-6B was from Pharmacia Fine Chemicals Inc., Piscataway, N.J. and chemicals for polyacrylamide gel electrophoresis were products of Bio-Rad, Richmond, California. Tissue culture supplies were purchased from Grand Island Biological Co. (Grand Island, N.Y.) or from Flow Laboratories (Rockville, MD.). Other chemicals were from local suppliers, and analytical grades were used where available.

Cell culture

The W1-L2 human lymphoblastoid cell line originated in 1968 [18]. Aspects of folic acid metabolism of these cells were discussed in earlier reports [3, 8, 17]. Some properties of the MTX-resistant sublines, and their origin, are discussed in [2] and [4]. The cells were grown in RPMI 1640 medium, supplemented with 10% foetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cultures were tested for mycoplasma contamination at monthly intervals. Stationary suspension cultures were set up at a starting density of 5×10^4 cells/ml, and harvested for enzyme studies in mid-log phase (750,000–1,000,000 cells/ml).

Enzyme assays

About 10^8 cells (per sample) were harvested by centrifugation at 400 *g* for 10 min, then washed by resuspending in Dulbecco's phosphate buffered saline and recentrifuging. The cell pellet was resuspended in 0·05 M tris chloride buffer, pH 7·2, and sonically disrupted (Bronwill "Biosonik" Ultrasonicator, setting 85, 20 sec at 0°C). Particulate material was removed by centrifugation at 105,000 *g* for 30 min, and the resulting supernatant fraction was used for enzyme assays. Thymidylate synthetase was measured isotopically by the method of Lomax and Greenberg [19]. Dihydrofolate reductase was assayed by the method of Mathews and Huennekens [20], modified as described previously [17], except that the assays were conducted at 37°C; these assay conditions employ deoxygenated buffer, but thiol reagents are not present, since these may alter the enzyme kinetic properties.

Purification of dihydrofolate reductase

Dihydrofolate reductase was purified to near-homogeneity by affinity chromatography on MTX-agarose gel columns. The MTX-

agarose was prepared as reported earlier [21] except that AH-Sepharose-6B was used as starting material. The purification method was as described in ref. [17].

Kinetic studies

The low values of the dihydrofolate reductase K_m for dihydrofolate and K_i for MTX necessitate special precautions during their determination, and appropriate statistical techniques for evaluation of data. These were described in detail in an earlier study [17].

Polyacrylamide gel electrophoresis

This was by the method of Davis [22], and gels were run at 4°C. The gels were stained for dihydrofolate reductase activity as described by Dunlap *et al.* [23].

RESULTS

The origin of the MTX-resistant sublines with which these studies were concerned has been described previously [2]. It is important to note that subline WR9.1 was derived from subline WR8.1 by increasing the maintenance level of MTX in the culture medium. These maintenance MTX concentrations, together with the ID_{50} concentrations of the drug for the different sublines, are noted in Table 1. Also shown in Table 1 are thymidylate synthetase activities of the cells. The activity of this enzyme was virtually identical in the parent line and the 2 MTX-resistant sublines. Since thymidylate synthetase has been implicated in some systems as a potentially significant site of action of MTX [24] we studied the kinetic properties of the enzyme from the parent strain of W1-L2 cells in some detail. For this purpose, 1,L-tetrahydrofolate was prepared by enzymatic reduction of dihydrofolate. One-hundred milligrams dihydrofolate was dissolved in 90 ml of tris chloride buffer, 0·05 M, pH 7·6. It was then reduced to 1,L-tetrahydrofolate as described by Huennekens *et al.* [25] using 40 IU of L1210 cell dihydrofolate reductase [21]. Figure 1 shows double-reciprocal plots of saturation curve experiments for 5,10-methylenetetrahydrofolate, in presence and absence of MTX. In these experiments the concentration of deoxyuridylic acid was maintained constant at 10^{-4} M, formaldehyde concentration was constant at 7×10^{-3} M, 1,L-tetrahydrofolate was added in varying amounts, and the actual concentration of 5,10-methylenetetrahydrofolate was calculated from the equilibrium

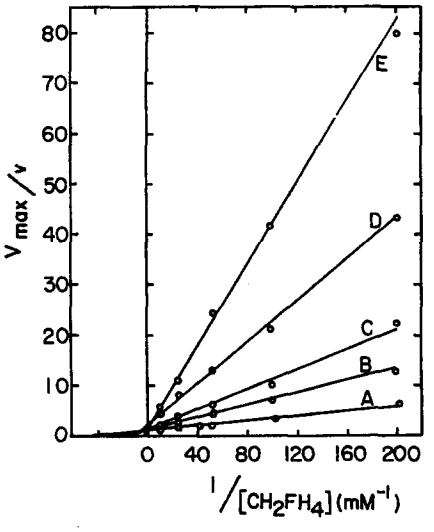

Fig. 1. Double reciprocal plots showing the dependence of W1–L2(WS) thymidylate synthetase activity upon the concentration of 5,10-methylenetetrahydrofolate and the competitive inhibition caused by MTX. A; no MTX; B, MTX 10 μM; C, MTX 20 μM; D, MTX 50 μM; E, MTX 100 μM. Experimental details are given in the text.

Table 1. Properties of W1–L2 cell sublines

Parameter	Sublines		
	WS	WR8.1	WR9.1
ID_{50} (μM)	0·013	2·2	6·8
Resistance (-fold)	(1)	170	523
Maintenance concn. of MTX (μM)	0	1·6	3·2
Thymidylate synthetase (IU/10^9cells)	0·025	0·027	0·024
Properties of dihydrofolate reductase:			
V_{max} (IU/10^9cells)	0·22	49·7	50·7
K_m for NADPH (μM)	3·1 ± 0·3	1·4 ± 0·2	N.D.
K_m for dihydrofolate (μM)	0·13 ± 0·02	2·3 ± 0·3	0·14 ± 0·04
K_i for MTX (pM)	7·3 ± 0·3	367 ± 48	6·8 ± 0·5
Turnover number (min^{-1})	1870	1610	1960

N.D. not determined. Values of kinetic parameters are followed by standard errors.

constant [26]. Other conditions were as described for the standard thymidylate synthetase assay. Results were calculated by the computer program "COMP" of Cleland [27]. The computed K_m for 5,10-methylenetetrahydrofolate of the thymidylate synthetase from parental strain W1-L2 (WS) cells was 23 ± 3 μM. MTX inhibited the enzyme competitively with 5,10-methylenetetrahydrofolate, and the K_i slope was 6·1 ± 0·8 μM; the intercept effect of MTX was very small. These parameters were based on a total of 72 experimental points.

Dihydrofolate reductase activities of the 3 sublines are listed in Table 1. Both the WR8.1 and WR9.1 sublines show an increase of about 230-fold in dihydrofolate reductase activity, relative to the parental cell strain. Subsequent experiments were concerned with the properties of the dihydrofolate reductase preparations isolated from the 3 sublines by affinity chromatography. The rate of dihydrofolate reduction was measured as a function of NADPH concentration, for the WS and WR8.1 enzymes. In each case a range of NADPH concentrations from 0·08 to 100 μM was studied, 32 experimental points for each determination. Results approximated closely to conventional hyperbolic saturation curves, with no indication of high substrate inhibition. K_m values for NADPH were calculated by non-linear regression analysis [28], and results are shown in Table 1. It may be seen that this parameter has decreased in the WR8.1 subline by more than a factor of 2, relative to the parental (WS) dihydrofolate reductase.

The determination of K_m values for dihydrofolate presents a number of practical problems; these were discussed in an earlier paper [17]. A range of dihydrofolate concentrations from 0·08 μM to 100 μM was studied, using a total of 28 points for each determination. Results are listed in Table 1. The K_m of dihydrofolate reductase for dihydrofolate was virtually identical to the normal value in subline WR9.1; in subline WR8.1, however, the binding of dihydrofolate was 18-fold weaker than normal.

The theoretical and practical aspects of the determination of K_i values for the interaction of dihydrofolate reductase and MTX were also discussed in ref. [17]. Figure 2(a) shows MTX titration curves for highly purified dihydrofolate reductase preparations from WR8.1 and WR9.1. Equal amounts of the 2 enzymes were used in these experiments, and equal amounts of dihydrofolate. Up to about 40% inhibition the curves are almost indistinguishable, but at higher inhibitor concentrations the different behaviour of the 2 enzymes becomes apparent. For example, at a total MTX concentration of 50 nM, the residual activity of the WR8.1 reductase is 3 times greater than that of the WR9.1 enzyme. Clearly the WR8.1 enzyme is binding the inhibitor less tightly than the dihydrofolate reductase from WR9.1 cells. Figure 2(b) is a linearized plot of the same data. These plots, for competitive inhibitors, have slope of $K_i(1+S/K_m)$, and the intercept at the ordinate gives enzyme concentration [17]. Thus, if K_m is already known, K_i may be calculated. However, a greatly preferable way of extracting K_i values for tight-binding inhibitors

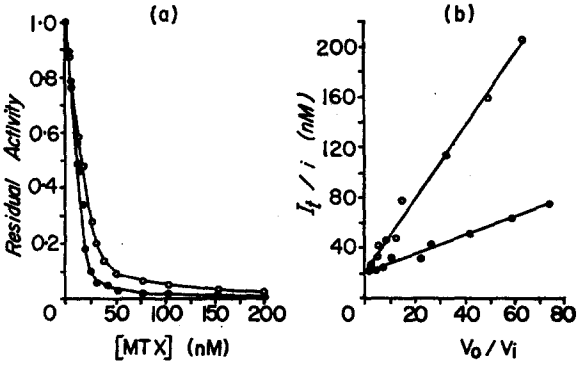

Fig. 2. (a). Titration of purified dihydrofolate reductases from WR9.1 (closed circles ●) and WR8·1 (open circles ○) with MTX. Enzyme concentration in each case was 20 nM and dihydrofolate concentration was 15 μM. NADPH was at 50 μM. (b) Linearized plot of the same data. v_0 is uninhibited velocity, v_i is velocity in presence of inhibitor. A total of 30 points were run for each enzyme, but for clarity not all are plotted. The K_i values plotted from the slopes of these graphs are:

WR 9.1, 0·747/(1+15/0·14) = 0·0069 nM
WR 8.1, 2·88/(1 ± 15 / 2·3) = 0·383 nM (equation is given in text).

is by the statistical method of Henderson [29]. The values listed in Table 1 were obtained by that method, as embodied in a computer program written by Hart [17]. As the results in Table 1 show, the K_i for MTX binding by the dihydrofolate reductase of WR8.1 cells is more than 50 times higher than that of the parental W1-L2(WS) cells; in the mutant subline WR9.1, derived from WR8.1 by selection at even higher MTX concentrations, the K_i has reverted to the original value.

To provide some indication of molecular size, dihydrofolate reductases from the 3 sublines were chromatographed through a 90cm × 3 cm^2 column of Sephadex-G100, eluted with Tris–chloride buffer, pH 7·2, 0·05 M, at 4°C. The column was standardised using ribonuclease A, chymotrypsinogen A, and ovalbumin. Enzyme from all 3 sources gave a

single peak on gel filtration, corresponding to a molecular weight of 22,500 ± 1000. Enzyme activity monitored following application of crude cell extracts to the Sephadex-G100 column also indicated a single peak, eliminating the possibility that minor dihydrofolate reductase components with different molecular weights were present in the cells but lost during the affinity chromatography procedure.

The isolation of essentially homogeneous dihydrofolate reductases made possible a determination of the enzymes' turnover numbers. These values are given in Table 1; they were measured at 30°C, to facilitate comparison with earlier results. No very marked differences in turnover number were seen between the sublines.

Figure 3 shows the results of polyacrylamide gel electrophoresis done with crude and highly purified dihydrofolate reductase preparations

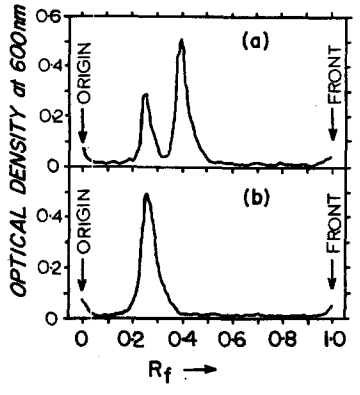

Fig. 3. Optical density scans of polyacrylamide gels stained for dihydrofolate reductase activity. (a) Crude cytosol preparation of WR8.1 (b) purified dihydrofolate reductase from WR8.1. Experimental details are given in the "Methods" section.

from WR8.1; the gels were stained for enzyme activity and then scanned. The crude extract showed peaks of reductase activity at R_f 0·26 (form I) and R_f 0·40 (form II). Peak II accounted for 63% of total activity. Following affinity chromatography only form I was present. Since the recovery of activity in the purification procedure was almost 90%, it is clear that form II was converted to form I in the course of purification. Polyacrylamide gel electrophoresis patterns for W1-L2(WS) and WR9.1 have been presented previously [2].

Finally, Fig. 4 illustrates the heat inactivation at 55°C, of the 3 dihydrofolate reductase preparations, in presence and absence of 0·2 mM NADPH. The rate of inactivation of WR9.1 enzyme was not greatly different from that of WS reductase, but the WR8.1 reductase was markedly less stable than the other two preparations.

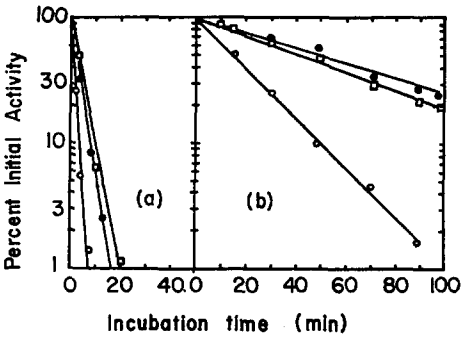

Fig. 4. Heat inactivation of purified dihydrofolate reductases. (a) No NADPH present; (b) 0·2 mM NADPH. Temperature was 55°. Symbols: □ WS; ○ WR8.1; ● WR9.1.

DISCUSSION

These studies have shown that a lymphoblastoid cell line, WR8.1, with acquired resistance to MTX, possesses a dihydrofolate reductase with grossly abnormal kinetic properties. Binding of MTX by this altered enzyme is over 50 times weaker than normal, binding of dihydrofolate is 18-fold weaker, and the K_i/K_m ratio is thus about 3-fold above the normal value in these cells. It is not known at what stage in the process of development of resistance these changes took place. If the mutation responsible for the changes was an early event in the acquired resistance process, cells possessing it would undoubtedly have a selective advantage. Earlier studies concerned with differences in intrinsic MTX resistance between cell strains showed that, when other factors such as total dihydrofolate reductase activity and rates of membrane transport were similar, cell lines with higher K_i values for MTX binding by dihydrofolate reductase were less sensitive to the drug. Figure 2 of the present paper compared MTX inhibition of equimolar amounts of WR8.1 reductase, and a dihydrofolate reductase with the normal K_i value (the enzyme used was actually from WR9.1, which has the same K_i as the WS reductase). Clearly the WR8.1 enzyme required considerably more drug to reach the same degree of inhibition. These studies have thus established that the production of kinetically abnormal dihydrofolate reductase is a possible mechanism of acquired MTX resistance in mammalian cells.

During the process of selection for MTX resistance, by growing cells in drug-containing medium, the total dihydrofolate reductase

activity of the cells increased greatly; both the resistant sublines examined in this study possessed about 230 times the original level of this enzyme. The turnover numbers of the enzymes were not greatly changed, indicating that the increase was due to the presence of more enzyme molecules, not to production of enzyme with greater catalytic potential.

When the WR8.1 subline was subjected to increased selective pressure, by further stepping up the drug concentration, the WR9.1 subline resulted. Table 1 of ref. [2] shows that the WR8.1 and WR9.1 sublines possessed identical activities of dihydrofolate reductase, and identical rates of membrane transport of MTX, yet WR9.1 was 3-fold more resistant to MTX than WR8.1. The present work has now shown that there is a biochemical difference between the 2 sublines, namely the kinetic properties of dihydrofolate reductase; however, on the basis of comparative reductase kinetics we might expect WR8.1 to be more, not less, resistant than WR9.1. This paradoxical state of affairs may be partly explained by consideration of Fig. 5. This diagram shows calculated activities of thymidylate synthetase and dihydrofolate reductase in the different cell lines, as a function of the free MTX concentration. The thymidylate synthetase curve (dotted line) is the same for all the sublines. The solid lines represent dihydrofolate reductase, which differs in amount and properties between the sublines. Consider curve A, representing the W1-L2(WS) cells. Thymidylate synthetase is normally the rate-limiting enzyme in the cycle of oxidation and reduction of folate cofactors (Table 1). However, as the MTX concentration increases, the potential activity of dihydrofolate reductase decreases, and when the free cellular MTX level exceeds 0·026 μM, the reductase becomes rate-limiting, and growth inhibition occurs. Line B represents a hypothetical cell line containing the normal activity of dihydrofolate reductase, but of the kinetically altered form found in WR8.1. This enzyme is more resistant to MTX inhibition, and the reductase curve does not cross the synthetase curve, and become rate-limiting, until a free MTX concentration of 0·083 μM is reached. Inhibition of the synthetase at this MTX concentration is negligible. When the dihydrofolate reductase, with normal kinetics, is increased 230-fold, as in WR9.1, the inhibition curve is given by line C. This situation differs radically from that of line A, in that thymidylate synthetase inhibition by MTX is now very pronounced. The synthetase, in fact, remains rate-limiting until a cellular free MTX concentration of about 30 μM is reached; at this point growth is inhibited to about 20% of the normal rate, and cells start to die. Curve D shows the situation in WR8.1. The altered properties of dihydrofolate reductase again shift the titration curve to the right, but this does the cell no good, because cell division is limited by thymidylate

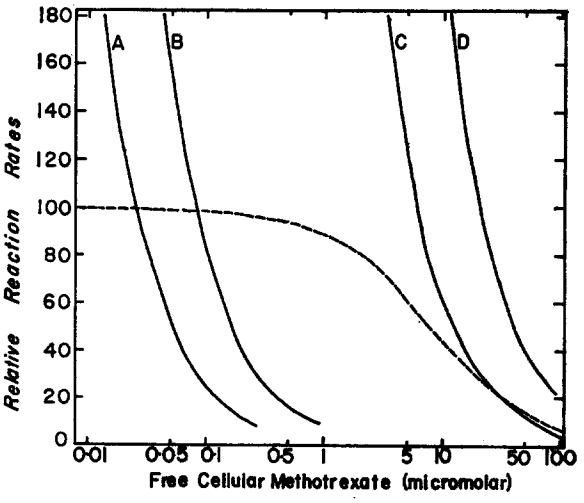

Fig. 5. Rates of thymidylate synthetase and dihydrofolate reductase reactions as a function of free intracellular MTX concentration. The thymidylate synthetase curve (broken line) is the same for all sublines; it was calculated using the competitive inhibition equation and the kinetic parameters reported in this paper. The 5,10-methylenetetrahydrofolate concentration was taken as 6 μM [30]. Saturation with dUMP was assumed since dUMP is known to accumulate in MTX-treated cells [31]. *Dihydrofolate reductase curves (solid lines) were calculated from the tight-binding competitive inhibition equation* [32] *and from the relationship:* $I_{free} = I_{total} - iE$ [32]. *Dihydrofolate (as the functionally active polyglutamate form) is known to accumulate in presence of MTX* [33, 17] *and we assumed a concentration of* 15 μM. *Other values used were: Curve A (WS cells)* $E_t = 0{\cdot}135\ \mu M$, $K_i = 7{\cdot}3 \times 10^{-12} M$; $K_m = 1{\cdot}3 \times 10^{-7} M$. *Curve B (hypothetical subline)* $E_t = 0.135\ \mu M$; $K_i = 3.65 \times 10^{-10} M$, $K_m = 2{\cdot}3 \times 10^{-6} M$. *Curve C (WR9.1 cells)* $E_t = 31{\cdot}6\ \mu M$, K_i *and* K_m *as for curve A. Curve D (WR 8.1 cells)* $E_t = 31{\cdot}6\ \mu M$, K_i *and* K_m *as for curve B. Other details are given in "Discussion" section.*

synthetase activity. Thus, in the highly MTX-resistant sublines, with greatly elevated dihydrofolate reductase, decreased affinity of the reductase for MTX provides no advantage to the cells.

In fact the results showed that possession of the altered dihydrofolate reductase by the highly resistant cells was actually a liability. This may be understood kinetically if we compare not, as in Fig. 5, the steady state, where equal free MTX concentrations are compared, but the situation in the cells before the steady state is reached; we thus compare the comparative effects of equal *total* MTX levels in WR8.1 and WR9.1. Assume $E_t = 31{\cdot}6\ \mu M$, and

dihydrofolate at 0·15 μM. Then, from the kinetic parameters listed in Table 1, and from the Goldstein equation [32] we may calculate free MTX levels in the cell, given the total cellular MTX content. If total MTX = 32 μM, then free MTX = 3·53 μM in WR8.1 and 0·93 μM in WR9.1. This 4-fold greater free MTX in the WR8.1 cells would give appreciably greater inhibition of thymidylate synthetase. The lower stability of the altered reductase, demonstrated in the present study, might also put the WR8.1 cells at a disadvantage.

To conclude, we have demonstrated the existence of a kinetically altered dihydrofolate reductase in cultured human cells. This mutation would have provided the cells bearing it with a selective advantage early in the development of resistance, but when the cells reached such a degree of MTX resistance that inhibition of thymidylate synthetase became significant, the mutation became a disadvantage, and disappeared. This property of conferring a selective advantage over a limited drug concentration range is not without precedent. For example, in mouse leukaemia cells subjected to gradually increasing amounts of MTX, transport mutants with altered K_t first appear, then, at high drug concentrations, disappear [13].

These studies also cast some light upon the importance of the low affinity sites of action of MTX. Of the various sites of action demonstrated for MTX, the inhibition of serine hydroxymethyltransferase may be dismissed as too weak to be of pharmacological significance. The importance of MTX inhibition of cellular uptake of 5-methyltetrahydrofolate is difficult to assess in the experimental system we used, since the culture medium contained folic acid, which utilises a different uptake route [2]. The possibility of MTX binding to low affinity sites on dihydrofolate reductase itself has been suggested [5]. This effect has been best characterised with the dihydrofolate reductase from *Lactobacillus casei* [34, 35]. MTX appeared to bind about 1000 times more weakly to the dihydrofolate reductase apoenzyme than to the enzyme–NADPH complex. However, since free reductase without NADPH cannot exert catalytic activity, binding of MTX to this apoenzyme is without pharmacological effect. Finally, as the present results have demonstrated, binding of MTX to thymidylate synthetase can be of importance. This mechanism of action of MTX would seem, though, only to be effective in the highly drug-resistant cells, with greatly elevated dihydrofolate reductase, where free intracellular MTX can accumulate to sufficient levels to exert significant inhibition of the thymidylate synthetase.

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Simultaneous Chemical Induction of MTV and MLV *in vitro*

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Abstract—*Latent endogenous mammary tumour virus (MTV) and latent endogenous mouse leukemia virus (MLV) were simultaneously chemically induced with concomitant cell morphological alteration in tissue-cultured baby mouse kidney cells (BMKC) derived from the low mammary tumour mouse strains BALB/c (substrains He.A and Crgl.A) and C57BL/Li.A. 3-Methylcholanthrene (MCA) was used with BMKC prepared from BALB/c/He.A and BALB/c/Crgl.A mice and 5-bromodeoxyuridine (BrdU) with BMKC prepared from C57BL/Li.A.*

Aggregates (pocks) of small rounded epithelioid cells appeared on the BMKC monolayers several weeks after chemical treatment. Cell lines produced from single pocks released MTV (B-particles) and MLV (C-particles) as found with electron microscopy and reverse transcriptase determinations. All isolated cell lines grew well when serum was omitted from the medium. In female syngeneic BALB/c/He.A mice MCA altered BMKC induced early (<300 days) mammary tumours and early (<200 days) leukemia. A cell-free extract from these chemically altered BMKC induced similar early tumours. Chemically non-treated control BMKC cultures did not show the described alterations and properties. The data support the conclusion that MTV can be present in "virus-free" mouse cells from which the virus can be chemically induced simultaneously with and in the same way as MLV.

INTRODUCTION

THE INDUCTION of latent endogenous C-type oncorna viruses in tissue cultured "virus–negative" normal and tumorigenic human and animal cells by halogenated pyrimidines [1, 2], chemical carcinogens [3, 4], mutagens [3], deprivation of an essential nutrient [5], aging [6], oncogenic DNA-virus [7], X-rays [8] and still other techniques [9] has been firmly established in recent years. Induction (activation) is defined here in the usual experimental meaning: the endogenous virus genome is made "expressed", so that the virus can be measured [9].

Chemically induced morphological alteration with concomitant induction of an endogenous C-type oncorna virus has been reported also [10].

For the intact animal hormonal and immunological activations may be added to the list of induction techniques for the C-type viruses [9].

On the contrary, the induction of the mouse-mammary tumour virus (MTV), the B-type oncorna virus, has only been described for the intact animal [11–13] and not yet for any type of tissue cultured mouse cells. We now report the simultaneous chemical induction of latent endogenous MTV and latent endogenous murine leukemia virus (MLV) with concomitant morphological transformation in tissue cultured baby mouse kidney cells (BMKC) prepared from the low mammary tumour mouse strains BALB/c (substrains He.A and Crgl.A) and C57BL/Li.A. Parts of this study have been published in abstract form [14, 15].

Accepted 26 October 1976.

MATERIAL AND METHODS

These have been described for the greater part in a previous publication [16].

Mice

BALB/c substrains He.A and Crgl.A have been described in more detail before [16]. Substrain He.A is known to contain endogenous MTV and MLV, which are expressed at a rather high age. Substrain Crgl.A has generally been considered to be "free of MTV" [17–19].

The C57BL/Li.A mouse strain has a very low spontaneous mammary tumour and a low

spontaneous leukemia incidence. B-particles could not be found in the spontaneous or radiation and chemically induced mammary tumour of these mice [12].

Mouse cells

All BMKC subcultures were made by pronase treatment after the cultures had reached 90–95% confluency [16]. So the first subcultures were prepared on the 4th day of *in vitro* transplantation: one primary culture giving three secondary cultures ($1 \rightarrow 3$). The second subcultures were made between the 11th and 17th day ($3 \rightarrow 1$) and the third subcultures between the 18th and 38th day ($1 \rightarrow 1$). The optimal transfer ratios were established in trial experiments.

Chemicals

The stock solution of 3-methylcholanthrene (MCA; Sigma) was made (100 μg/ml) in an acetone-dimethylsulphoxide (DMSO) mixture (50:50, v/v). The final concentration of MCA was 10^{-3} μg/ml and of each of the organic solvents $5 \cdot 10^{-4}$ %. In preliminary experiments we had found this a "non-cytotoxic" dose of MCA when added from the third up to and including the 6th day. The controls in the MCA induction experiments got only the same amount of the acetone–DMSO mixture. The stock solution of 5-bromo-2-deoxyuridine BrdU; Sigma) was made in H_2O. The final concentration of BrdU in the medium was 30 μg/ml.

RESULTS

Cytotoxicity of 3-methylcholanthrene for BALB/c/He.A–BMKC monolayers

In a typical experiment 0, 10^{-3}, 10^{-2} and 10^{-1} μg/ml MCA were added respectively to the media of 4 groups, each consisting of 3 BMKC cultures, from the third up to and including the 6th day. On the 7th day the estimated confluencies were 40–50%, 40–50%, 30–40% and ± 30%. On the 9th day the percentages were: 80–90%, ± 80%, 30–40% and ± 30%. And on the 10th day: ± 90%, ± 90%, ± 60% and 20–30%.

In another experiment 10^{-3} μg/ml MCA was added to the media of 3 groups each consisting of 3 BMKC cultures. In group I from the third up to and including the 6th day (3rd–6th day), in group II from the 7th–10th day and in group III from the 3rd–10th day. On the 11th day the second subcultures were made and on the 15th day the confluencies were estimated.

Control (no MCA): ± 50%, I: ± 50%, II: ± 40%, III: ± 10%. We chose 10^{-3} μg/ml as the MCA concentration for the chemical induction experiments and applied it to the monolayer cultures from the third up to and including the 6th day. From the preliminary cytotoxicity experiments it could be concluded that the application of 10^{-3} μg/ml MCA during a longer (3rd–10th day) or later (7th–10th day) period and higher ($> 10^{-3}$ μg/ml) MCA concentrations were clearly cytotoxic as expressed in cell growth retardation.

Morphological transformation of BALB/c–BMKC with concomitant induction of MTV and MLV by 3-methylcholanthrene

After 3–6 weeks MCA treatment induced the formation of macroscopically visible 3-dimensional aggregates (pocks) of small rounded cells on the BMKC monolayers derived from BALB/c/He.A and from BALB/c/Crgl.A (Table 1; Fig. 1–3). The morphologically altered cells and the pocks appeared microscopically very similar to the pocks induced by exogenous mammary tumour virus (MTV) on BMKC-cultures [16]. Here too, cell lines could easily be derived from individual pocks. The cell lines grew rapidly in our standard medium with and without serum. With May–Grünwald–Giemsa staining the MCA-transformed BMKC showed a brilliant dark blue colour; as did the MTV transformed BMKC [16]. The cell lines formed epithelioid monolayers on which pocks were formed again. The MCA transformed BMKC cultures produced and released both B- and C-particles (Figs. 4, 5). When these cells were incubated with rabbit anti-MTV serum followed by incubation with ferritin labeled goat anti-rabbit IgG as described in [20] the B-particles were labeled but the C-particles produced by the same cells were always negative (Fig. 4).

The oncogenicity of cell line A3, derived from a single pock of MCA induced and morphologically altered BMKC, prepared from BALB/c/He.A mice, is shown in Table 2. Each of the three differently with cell material inoculated groups of mice: (1) intraperitoneally (i.p.) with A3 cells, (2) subcutaneously (s.c.) with A3 cells, (3) i.p. with an A3 cell extract), were divided, for technical reasons, in four nearly equally large subgroups: a–d. Subgroups (a) were inoculated with A3 cells of the 16th subculture (144 days after visible *in vitro* transformation). Subgroups (b) were inoculated with A3 cells of the 19th subculture (164 days). Subgroups (c) got A3 cells of the 21st subculture (178 days) and subgroup (d) of the 23rd subculture (191 days). So the three

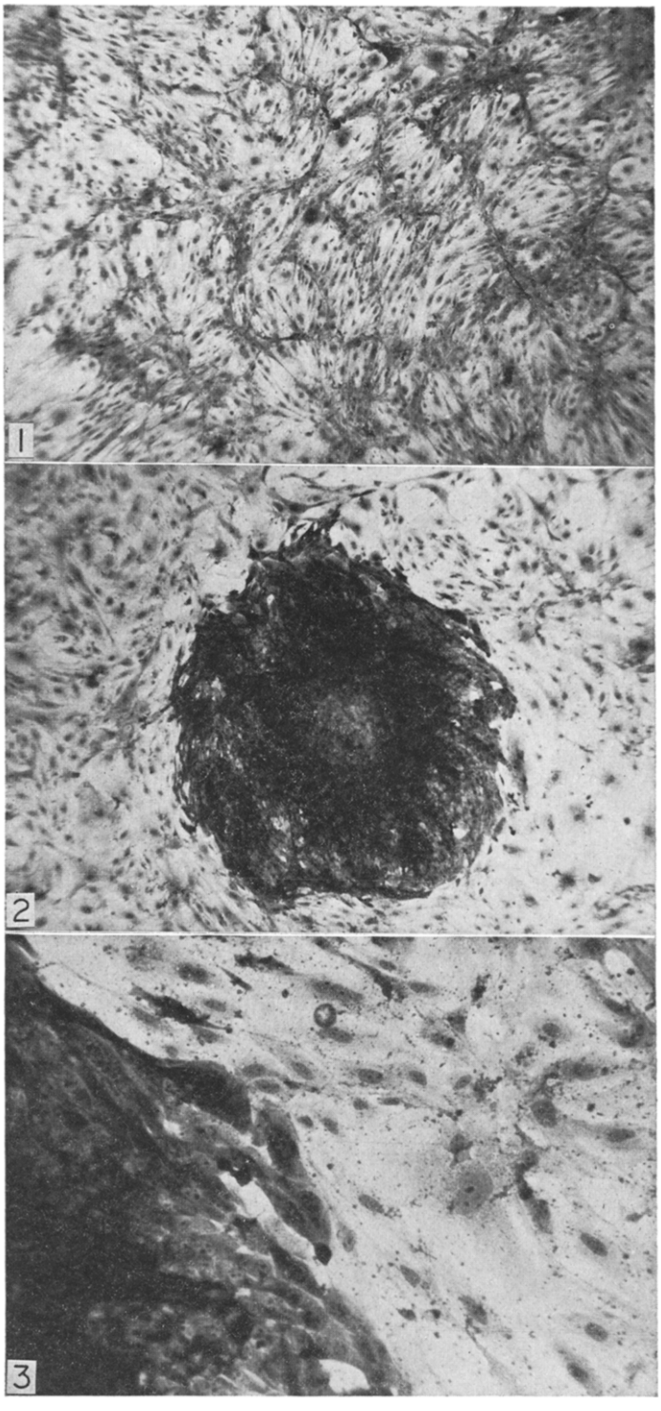


Fig. 1. *BMKC monolayer derived from BALB/c/He.A mice. Third subculture,* 50 *days after starting primary culture.* × 30.

Fig. 2. *Methylcholanthrene (MCA) induced focus of altered cells (pocks) in BALB/c/He.A—BMKC monolayer of the same age as in Fig.* 1. × 30.

Fig. 3. *Enlargement of edge of the same pock as in Fig.* 2. × 120.

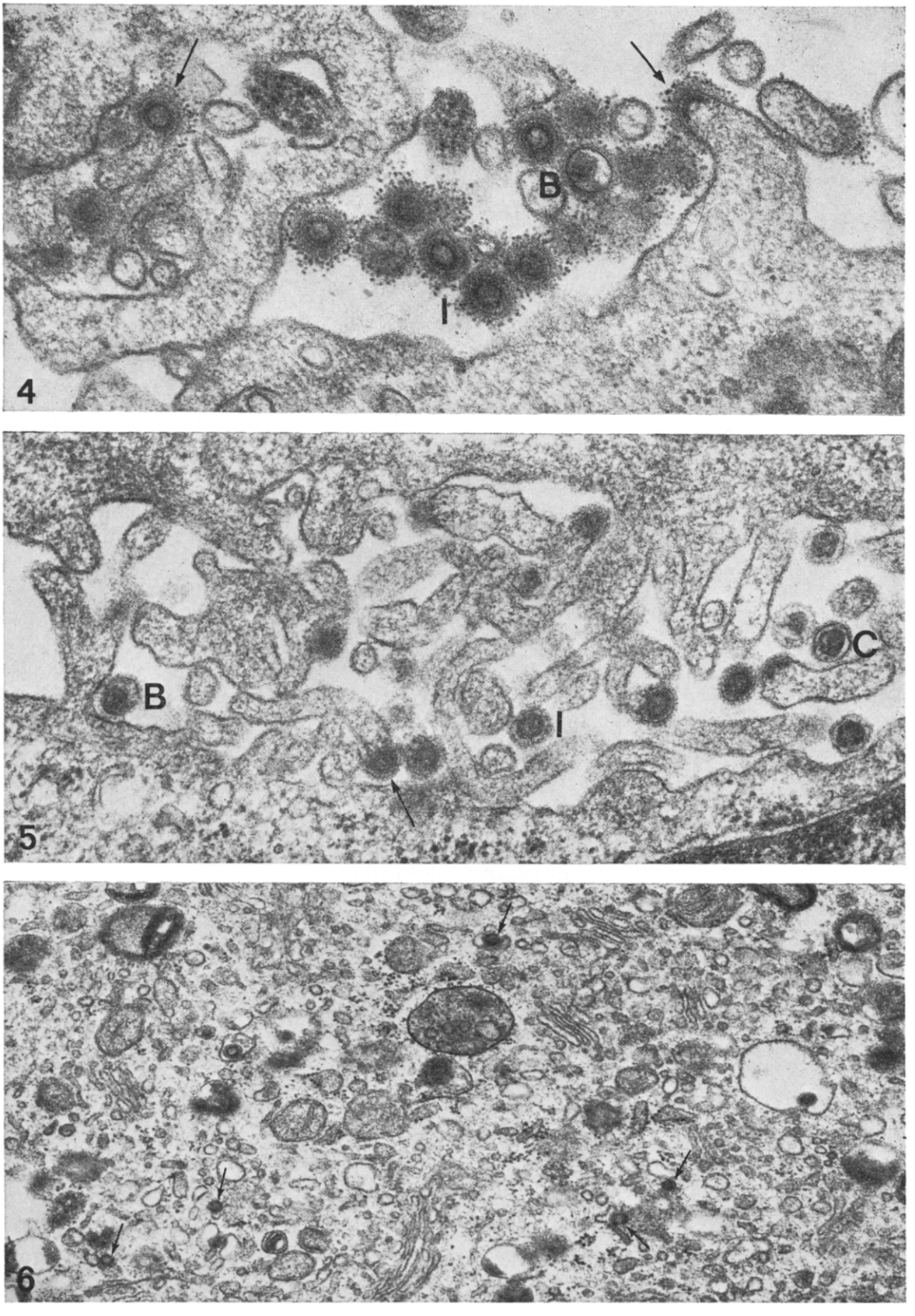
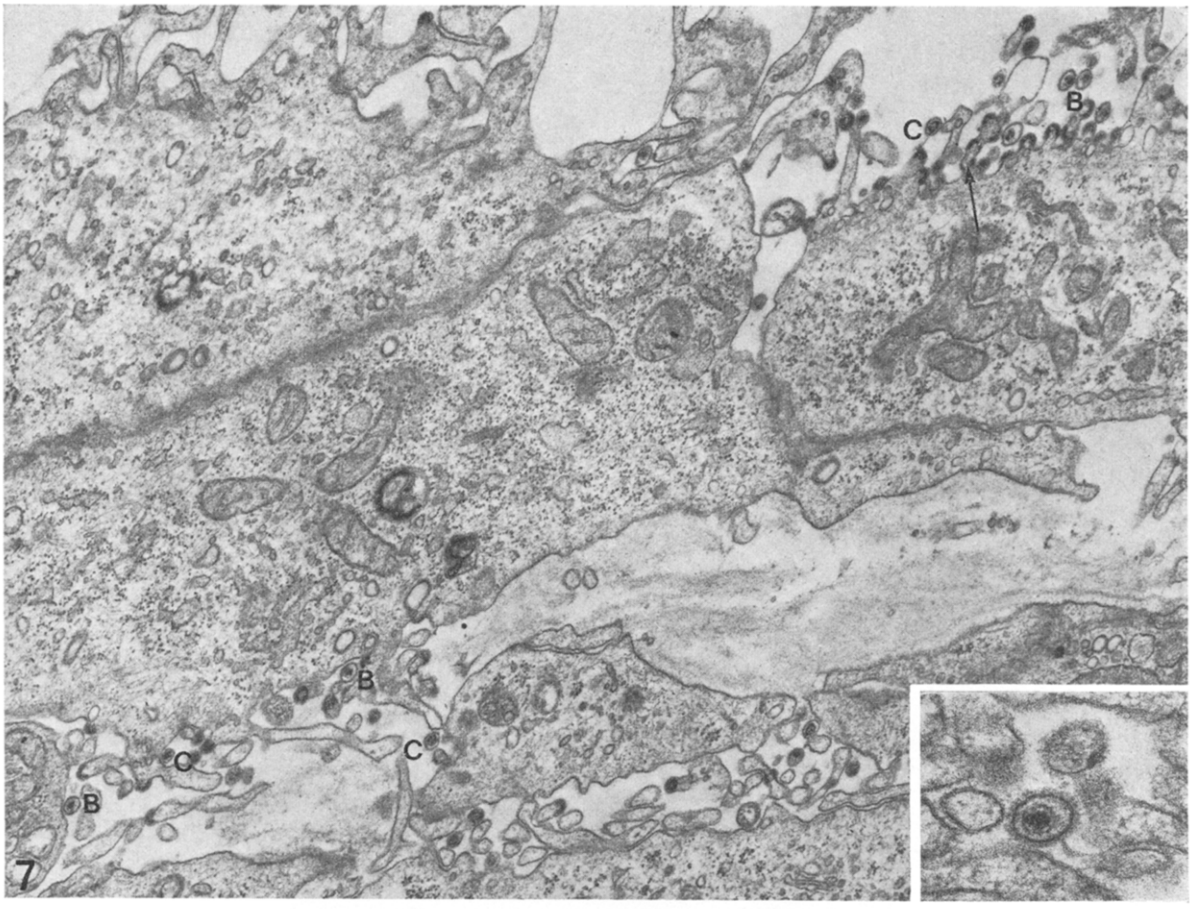


Fig. 4. *MCA transformed BALB/c/He.A—BMKC culture incubated with rabbit anti-MTV serum. Budding (arrows), mature (B) and immature (I) B-particles are labeled.* × 76,000.

Fig. 5. *MCA transformed BALB/c/Crgl.A—BMKC culture. B (B), C (C), immature (I) and budding (arrow) particles are shown.* × 66,500.

Fig. 6. *BrdU transformed C57BL/Li.A—BMKC culture. Intracytoplasmic A particles (arrows) are present.* × 37,000.



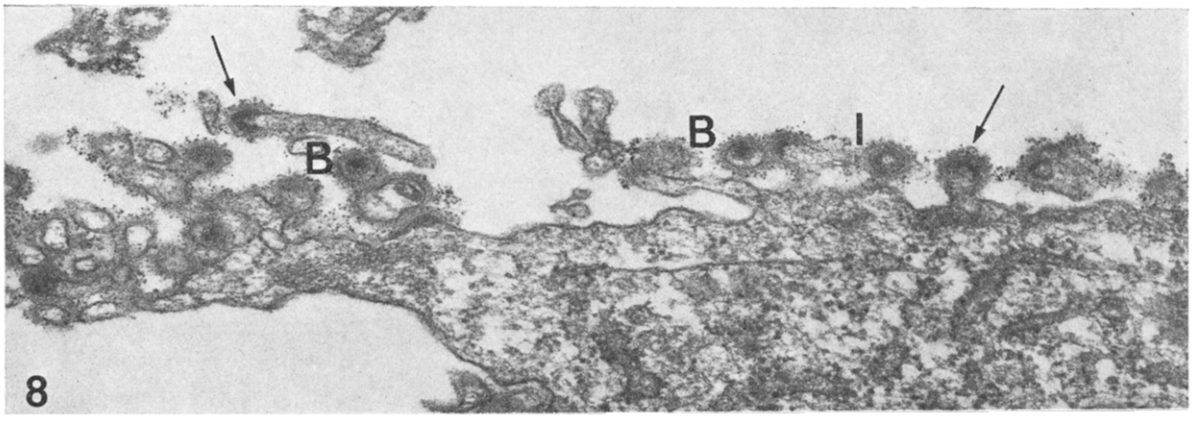


Fig. 7. BrdU transformed C57BL/Li.A—BMKC culture. B (B) and C (C) particles were abundant. Several virions are budding from a common stalk (arrow). × 27,500. Inset. *B particle at higher magnification showing an eccentric nucleoid surrounded by an intermediate layer and an envelope with surface projections.* × 76,000.

Fig. 8. BrdU transformed C57BL/Li.A—BMKC incubated with rabbit anti-MTV serum. Budding (arrows), mature (B) and immature (I) B particles are labeled. × 47,500.

Table 1. *Morphological transformation of BALB/c baby mouse kidney cell cultures by 3-methylcholanthrene (MCA) and mammary tumour virus (MTV(S))*

BALB/c substrain	Added* MCA	Added* MTV	No. of foci	No. of cultures containing transformed foci/No. total	Foci detected on day
/He.A	–	–	0	0/7	—
	+	–	8	3/6	50–52
	–	+	3	2/7	49–53
	+	+	55	8/8	20–53
/Crgl.A	–	–	—	0/5	—
	+	–	50	5/5	22

*For details see text.

Table 2. *Tumour incidences in ♀ BALB/c/He.A mice inoculated with 3-methylcholanthrene induced syngeneic BMKC (cell line A3)**

No. of mice	Inoculation Amount (cells)†	Inoculation Route	Mammary tumours <300 days %	Mammary tumours <300 days Age (days)	Mammary tumours >300 days %	Mammary tumours >300 days Age (days)	Leukemia %	Leukemia Age (days)
18	—	—	0	—	33	383 ± 40	44	298 ± 94
17	1×10^6	i.p.	24	206 ± 47	0	—	88	188 ± 40
19	$0{\cdot}4 \times 10^6$	s.c.	53	215 ± 35	11	309 ± 8	68	210 ± 50
16	10×10^6 cell free	i.p.	19	175 ± 14	0	—	94	159 ± 26

*Cell line A3 was derived from a single pock of MCA induced and morphologically altered BMKC prepared from BALB/c/He.A mice. Forty days after MCA treatment the pock became visible on the BMKC monolayer.
†Subculture 16, 19, 21 and 23 of the A3 cells were used for inoculation. For details see text.

differently inoculated groups of animals were treated with A3 cells of practically the same average *in vitro* age. The A3 cells induced early (< 300 days) mammary tumours and early (< 200 days) leukemia along both routes of infection, (i.p. or s.c.). An i.p. introduced A3 cell extract gave similar early tumours (Table 2).

Determinations of the RNA-dependent DNA polymerase activities were carried out in the high speed pellets (Spinco L2, rotor No. 30; 40,000 *g* for 90 min) of the 24 hr media of A3 cell cultures (Table 3).

The ratio of the Mg^{2+} stimulated (largely specific for MTV) and the Mn^{2+} stimulated (largely specific for MLV) activity was 1·3 in the 13th subculture (118 days) and 0·5 in the 23rd subculture (191 days). Thus the B-particle production was lowered as compared with the C-particle production upon *in vitro* aging of the A3 cell line. This is in general agreement with earlier electron microscopic observations [20]. In a previous publication we described the morphological transformation and concomitant production of B- and C- particles in BALB/c–BMKC by exogenous MTV [16]. Now it appeared that MCA and exogenous MTV had a synergistic effect on the morphological alteration (pock formation) and concomitant production of MTV and MLV in BMKC derived from BALB/c/He.A mice (Table 1). MCA was added as described (Methods) and exogenous MTV dilution 10^{-3} was adsorbed on the BMKC monolayers for 2 hr on the 3rd day [16].

Table 3. *Cation preferences for RNA directed DNA polymerase activities in media of A3 cells in two different subcultures*

Subculture	Incorporation Mg^{2+}†	Counts/min of d-GTP-^{3}H in presence of Mn^{2+}†	Mg^{2+}:Mn^{2+}‡
13*	22·602	16·974	1·3
33	4·458	9·255	0·5

*The A3 cells of subcultures 13 and 23 had been kept *in vitro* for 118 and 191 days after visible transformation.
†8 mM Mg^{2+} or 0·3 mM Mn^{2+}.
‡Ratio of counts/min of d-GTP-^{3}H incorporation.

The oncogenicity of the MCA induced MTV and MLV in BALB/c/Crgl.A–BMKC is being

investigated in syngeneic female mice. The control BMKC cultures from both BALB/c substrains did not produce B- or C-particles.

Morphological transformation of C57BL/Li.A–BMKC with concomitant induction of MTV and MLV by 5-bromo-2'-deoxyuridine (BrdU)

In a BMKC culture (one out of three) derived from C57BL/Li.A mice and treated with BrdU during 9 days (3rd–11th day) we got a single pock of small rounded cells 25 days after *in vitro* transplantation.

The pock and the morphologically altered cells appeared microscopically very similar to the pocks induced by MCA in BALB/c–BMKC cultures. A rapidly growing cell line could be derived from the pock. The chemically altered epithelioid cells grew equally well in the standard medium without serum.

The cells produced intracytoplasmic A-, B- and C-particles (Fig. 6, 7). The B-particles were labeled after incubation with rabbit anti-MTV serum followed by incubation with ferritin labeled goat antirabbit IgG (Fig. 8). The oncogenicity of the virus producing cells is being investigated with our colleague Dr. A. Timmermans. We did not yet succeed in inducing pocks on C57BL/Li.A–BMKC monolayers with MCA.

DISCUSSION

The described experiments showed (1) that tissue cultured baby mouse kidney cells (BMKC) derived from the low mammary tumour mouse strains BALB/c (substrains He.A and Crgl.A) and C57BL/Li.A contain the genetic information for MTV and MLV in a suppressed form and (2) that these BMKC can be chemically induced to produce and release simultaneously MTV virions and MLV virions. The BMKC derived from all three strains were explanted *in vitro* at the age of 5–7 days when no expression of endogenous MTV was found yet in the animals [21]. The age of 5 days is the lowest up to the present that B-particles could be found or have been made visible in organs of any mouse strain [22]. The induced MTV and MLV in BMKC derived from BALB/c/He.A were biologically active in syngeneic female mice (Table 2).

The induction of B-particle and C-particle production in BMKC derived from mouse strain C57BL/Li.A and substrain BALB/c/Crgl.A is even more striking as these mouse strains were not yet known to carry the information for complete MTV virions [13, 17–19, 23, 24].

Many studies have led to the solidly based conclusion that endogenous C-type oncorna viruses can be vertically transmitted as chromosomal elements [25–29]. Avian and murine systems have been studied most thoroughly in this respect. The viral genetic information of the chromosomes may vary from being unexpressed ("virus-free" state) via partially to fully expressed in the cells. Next to the discovery of the RNA-directed DNA polymerase (RDDP; reverse transcriptase) activity in oncorna virions [30] the inducibility of C-type oncorna viruses in tissue cultured "virus-free" cells [1–9] has contributed greatly to the present day notion of the existence of integrated viral genes from C-type oncorna viruses in the host cell chromosomal complement similar to the lysogenic state of bacteria. The development and acceptance of an equal lysogeny model for endogenous MTV (B-type morphology) paralleled more or less the history of the lysogeny model for C-type viruses [25, 28, 31–35]. Bentvelzen continuing Mühlbock's MTV transmission studies [32] with crosses of GRS (GR) and other mouse strains and their progeny concluded that a mammary tumour controlling factor was present as a single gene locus in the genome of the GR host [25, 33]. To explain this fact he favored a hereditary transmission model in which a DNA copy of MTV-RNA is permanently integrated in the genome of the GR host and is transmitted as a Mendelian factor (B.'s "germinal provirus"). A similar explanation was extended by him to the endogenous MTV of C3Hf mice [33] and comparable *f* mouse strains [35]. He did not completely reject a model in which a dominant susceptibility gene would cause extreme susceptibility of mice with the GR genome to small amounts of not integrated omnipresent (including the seminal fluid) MTV but considered this as "less economical" [33].

Nandi and Helmich repeated and extended the genetic studies of congenital MTV transmission in GR mice. Nandi came also to the conclusion that the genetic studies alone could not discriminate between integrated provirus genes and host susceptibility genes [36, 37].

We believe that the *in vitro* chemical induction of MTV in kidney cells derived from baby BALB/c mice (especially BALB/c/Crgl.A mice) and those derived from C57BL/Li.A mice resulting in the production and release of complete MTV virions, supports the integrated provirus theory for endogenous MTV [33]. The more so as with previous immunofluorescence tests and a nucleic acid hybridization assay, using a tritiated single-stranded DNA

probe complementary to a part of the MTV RNA genome, no MTV could be found in kidneys of young mice belonging to BALB/c/He.A and C57BL/Li.A strains [21, 38].

The existence of integrated endogenous MTV does of course not rule out another part played by host susceptibility (permissive) genes in MTV expression and biological activity [33–37, 39].

The inoculation of MCA induced MTV and MLV producing BALB/c/He.A–BMKC in syngeneic female mice caused early mammary tumours and early leukemia but no tumours on the site of injection (Table 2). The underlying mechanisms may have been the induction of interferon and of specific immunity to the chemically transformed cells without neutralizing all escaped oncorna virus particles on their way to target cells [40].

The A3 cell line, a chemically transformed BMKC derived from BALB/c/He.A, gradually lowered its B-particle production *in vitro* (Table 3). This is in general agreement with electron microscopic observations [20]. Apparently, only during a rather limited period after chemical induction, the BMKC can produce and release B-particles in comparatively large quantities. A similar gradual decrease of B-particle production was found with BMKC induced *in vitro* by exogenous MTV [16] and when B-particle producing mammary tumours of the mouse were explanted into tissue culture [41].

It may be an innate incapability of the MTV producing cells to produce permanently large quantities of B-particles under the presently used tissue culture conditions. One could also think of some kind of intracellular interference of ubiquitous MLV.

The endogenous MLV which is chemically and simultaneously induced with endogenous MTV in experiments with BMKC derived from BALB/c/He.A mice is likely more closely related to BALB/c–MLV No. 1 than to BALB/c-MLV No. 2 of Aaronson and Stephenson [42].

The BMKC cell lines derived respectively from BMKC transformed by exogenous MTV (previous study [16]) and from BMKC transformed by chemicals (MCA and BrdU) have several properties in common.

Both groups have a small and epithelioid morphology, grow equally well in serum—enriched and serum—less medium, produce and release simultaneously MTV virions and MLV virions (both viruses are biologically active in both groups of induced BMKC derived from BALB/c/He.A mice) and gradually lose their B-particle producing capability by aging of the cell lines *in vitro*. Further, exogenously applied MTV and MCA can act synergistically in the morphological alteration of BMKC and the simultaneous induction of MTV and MLV (Table 1). The chains of intracellular reactions following the primary triggering by the chemical and by exogenous MTV and resulting in the production and release of B- and C-particles have apparently several common links from close to the level of gene derepression [28].

However, the final production and release of B-particles and the final production and release of C-particles are not coupled as the relative virus yields change with the *in vitro* age of the host cells (Table 3).

Relevant to this study are the recent results of Lazar *et al.* This team described the chemical induction (with 5-iododeoxyuridine) of an ecotropic endogenous carcinogenic oncorna virus in an epithelioid cell line derived from pooled C57BL/6 mouse embryos [43]. These C57BL/6 mice have like the C57BL/Li.A mice in the present study a very low incidence of spontaneous tumours. The induced epithelial cells and the cells secondarily infected by Lazar's virus became morphologically transformed *in vitro* and produced carcinomas at the site of inoculation in syngeneic mice. It is interesting to note that the observed virus particles had a C-type morphology and caused syncytia formation in the rat sarcoma cell line XC [43].

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Cytological and Cytochemical Analysis of Two Mouse Cancer Cell Lines. Caryotype, Number of Nucleoli, DNA, RNA and Protein Contents

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Abstract—*Ehrlich ascites tumour cells (hyperdiploid strain ELD and hypertetraploid strain ELT) propagated in the mouse and TIII mammary carcinoma cells (strains TIII$_t$ and TIII$_r$) cultivated* in vitro *are analysed with cytological and quantitative cytochemical methods. It is shown that, in these cancer cell lines, the number of nucleoli and the DNA, RNA and total protein contents are related to the number of chromosomes. These cellular parameters are also analysed during the cell cycle.*

INTRODUCTION

IN THE present work, two mouse cancer cell lines are analysed with cytological and cytochemical methods: Ehrlich ascites tumour cells growing in the mouse peritoneal cavity and cells from a mammary carcinoma (TIII line) cultivated *in vitro*. Two strains from each of these cell lines are studied here in order to detect eventual relations between the caryotype and various cellular parameters: cell volume, number of nucleoli, nucleic acid and protein contents. The analysis of the two latter was performed with quantitative cytochemical methods which are particularly valuable because measurements in individual cells can be realized.

The results of the present investigation demonstrate that these cellular parameters are in fact related to the number of genomes in these two cancer cell lines. Nucleoli and nucleic acid and protein contents are also studied in these cells, during interphase (preparation for mitosis) and during cell division.

MATERIAL AND METHODS

1. *Cancer cell lines*

(a) *Mouse Ehrlich ascites tumour cells.* Hyperdiploid strain ELD and hypertetraploid strain ELT; these strains have been supplied by the Karolinska Institute (Stockholm) and are propagated in the peritoneal cavity of C57 Bl mice (Laboratory of pathological Anatomy, University of Liège).

(b) Cells from a *mouse mammary carcinoma* are continuously cultivated *in vitro* (line TIII) by Professor J. A. Thomas (Cell Biology Laboratory and Cell Physiology Center, Paris) who very kindly gave us strain TIII$_t$ and strain TIII$_r$ cells [1, 2].

2. *Cytological and cytochemical methods*

(a) Cell staining by von Möllendorff haematoxylin.

(b) Specific staining of *nucleoli* by toluidin blue after neutral deoxyribonuclease (DNase) digestion [3]. The mean number of nucleoli per cell was calculated from results obtained by observing 100–200 cells in each case.

(c) *Caryotype* analysis after treatment with an hypotonic solution and Unna blue staining.

(d) *Cell volume* measurements with an electronic counter (Coulter counter).

(e) *DNA, RNA and protein measurements* in individual cells by quantitative cytochemical methods; 50–100 cells in interphase or in mitosis were measured in each case.

DNA content: measured by absorption cytophotometry after Feulgen reaction (3·5 HCl hydrolysis at 37°C; Barr and Stroud GN2 microdensitometer, wavelength: 543 nm). Smears containing mouse leukocytes were stained under standard conditions in order to

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measure the mouse DNA diploid amount by cytophotometry. Values found and expressed in arbitrary units were converted into absolute values (pg) by using the mouse DNA diploid amount [4] measured by biochemical methods (5·5 pg).

RNA content: measured by absorption cytophotometry after neutral DNase digestion and gallocyanin–chrome alum staining [5]; GN2 microdensitometer, wavelength 550 nm; the results (total cellular RNA content) are expressed in arbitrary units. All the cells to be measured were stained simultaneously.

Total cell protein content: measured by absorption cytophotometry after naphtol yellow S staining [6]: GN2 microdensitometer, wavelength 435 nm; the results are expressed in arbitrary units.

Total cell dry mass: measured by scanning and integrating micro-interferometry (Vickers M86); the results are obtained in absolute amounts (pg) and essentially represent the total cell protein content [7].

RESULTS

A. *Mouse Ehrlich ascites tumour*

The origin of this line is a mouse mammary carcinoma [8, 9]. It is propagated as an ascites tumour in many laboratories.

Our ELD cell strain is *hyperdiploid* (45 chromosomes); our ELT cell strain is *hypertetraploid* (90 chromosomes, see Table 1). The normal mouse caryotype contains 40 chromosomes as it is well known. The *DNA content*, measured by cytophotometry after Feulgen reaction in cells fixed at the beginning of interphase, is hyperdiploid for ELD and hypertetraploid for ELT, when compared to the mean DNA amount of leukocytes from the same animal. The *modal cell volume* is 700 μm^3 and 1220 μm^3 for ELD and ELT respectively.

The mean *number of nucleoli* is 1·69 ± 0·58 for ELD and 3·38 ± 0·92 for ELT (Table 1). In some cells, only one large nucleolus is observed; this fact is usually due to nucleolar fusion. In both strains, large cells at the end of interphase contain particularly prominent nucleoli resulting from nucleolar growth during preparation for mitosis [3, 10].

The total *cellular RNA content* was measured by absorption cytophotometry in visible light. In both Ehrlich cell strains, large intercellular variations of the RNA content are observed. The RNA amounts are situated between minimal and maximal values; the ratio between the two latter is 1:2. The first ones are found in small post-mitotic cells fixed at the beginning of interphase; the second ones are found in mitotic cells and in large interphase cells. Thus these facts demonstrate that the total cellular RNA content doubles during preparation for mitosis, as DNA do. Furthermore, the RNA content in ELT cells is quite twice as high as in ELD cells (Table 1). The same conclusions are drawn from *total cell protein* measurements (by absorption cytophotometry or by micro-interferometry) in ELD and in ELT cells (Table 1). The protein content is doubled during interphase before mitosis starts; the protein content is twice as high in ELT as in ELD cells [11–14].

B. *TIII mouse mammary carcinoma*

The *caryotype* of various strains of this tumour (which are cultivated *in vitro*) was established by Thomas and his collaborators [2]. The modal chromosome number is around 60 for $TIII_r$ and around 100 for $TIII_t$ (Table 1).

We measured the *DNA content* by absorption cytophotometry in these two strains. $TIII_r$ is hypotetraploid, $TIII_t$ is hypertetraploid as to DNA (Table 1). These results thus completely agree with the caryotypes of these strains. Simultaneously stained mouse leukocytes were

Table 1. *Cytological and quantitative cytochemical analysis of four mouse tumour cell strains. Values related to DNA (in pg), RNA (in arbitrary units, a.u.) and to dry mass (in pg) are mean amounts found for cells fixed just after mitosis (in post-telophase)*

		Modal number of chromosomes	DNA content pg	Mean number of nucleoli	RNA content a.u.	Total cell dry mass pg
Ehrlich tumour	ELD	45	6	1·7	8·4	158
	ELT	90	12	3·4	16·0	302
Mammary carcinoma	$TIII_r$	60	7·9	2·0	8·4	238
	$TIII_t$	100	13·4	3·0	16·8	384

used as a reference for the mean DNA diploid amount. It is to be noted that the ratio $TIII_t/TIII_r$ is nearly identical as regards DNA and the modal chromosome numbers.

The *number of nucleoli* (Table 1) is $2{\cdot}06 \pm 1{\cdot}03$ for $TIII_r$ and $3{\cdot}05 \pm 1{\cdot}06$ for $TIII_t$. In some cells, nucleolar fusion is noted; nucleoli with increased volume are observed in late interphase cells.

The results of our absorption cytophotometry measurements in interphase or mitotic $TIII_t$ and $TIII_r$ cells clearly show that the *cellular RNA content* is doubled during preparation for mitosis. Furthermore (Table 1), the RNA content is twice as high in $TIII_t$ cells as in $TIII_r$ cells. Similar conclusions can be drawn from the results of our *total cell protein* measurements performed by cytophotometry or by interferometry in $TIII_t$ and $TIII_r$: the total cell protein content is doubled before mitosis starts and cell protein amounts are quite proportional to the chromosome number in each strain (Table 1).

DISCUSSION

Cell lines derived from *mouse mammary carcinomas* and actively growing *in vivo* or *in vitro* are analyzed with cytological and cytochemical methods. Ehrlich tumour cells are propagated in the mouse peritoneal cavity; TIII tumour cells are cultivated *in vitro* but are oncogenic when inoculated into young mice [1]. These two lines are compared as far as various cellular parameters are concerned. For both types of tumours, strains with different caryotypes are analyzed, thus allowing us to study an eventual relation between the number of chromosomes and these parameters.

Our quantitative cytochemical measurements demonstrate that the DNA, the RNA and the total protein contents of the cells are doubled before mitosis starts and are distributed in quite equal amounts between the daughter-cells. These facts are not different from those observed in other cancer cell lines [7] or in non cancer cells (e.g., fibroblasts *in vitro* [15]). During preparation for mitosis, the number of nucleoli is not modified but their volume and dry mass tend to double [3, 10]. This fact is also observed in the presently studied cancer cell strains.

In each of our cancer cell lines, the DNA content is clearly proportional to the chromosome number. This fact agrees with a classical notion. Our results also show that the cellular RNA content (essentially ribosomal RNA, [5]) and the total cellular protein content are also related to the chromosome number. These cell parameters thus seem to depend on a genetic control. The number of nucleoli is also increased with the number of genomes. This can be explained by the fact that the nucleoli are formed by specific chromosomes bearing a nucleolus organizer. However, nucleolar fusion can occur in some cells which then contain only one nucleolus; the latter is particularly large.

In a previous work [16], we compared ELD to ELT cells (45 and 90 chromosomes respectively) at the ultrastructural level. No morphological differences were noted. An analysis by morphometry showed that *in both strains* the volume occupied by the nucleus and by the mitochondria is 40% of the cell volume and 16% of the cytoplasm volume respectively. In fact, the cell volume is quite twice as large in ELT as in ELD. So, it can be stated that, in Ehrlich tumour cells, the volume of the nucleus and of the cytoplasm and the volume and the number of mitochondria are nearly doubled when the number of chromosomes is doubled. As far as the nucleoli are concerned [17], these organelles (perinucleolar chromatine being excluded) occupy 20% of the nucleus volume as well in ELD as in ELT cells.

When comparing Ehrlich to TIII carcinoma cells, it is observed that the values found for different cell parameters are of the same order of magnitude but, in general, these values are closely related to the chromosome number in each case. In fact, these cells all originate from mouse mammary carcinomas.

Concerning the cell cycle and the cell parameters studied here, no fundamental differences have been detected in these cell strains when they are compared to actively multiplying non cancer cells.

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Effective Antitumour Conjugates of Alkylating Drug and Antibody Using Dextran as the Intermediate Carrier

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Abstract—*The use is described of water-soluble dextran as an intermediate carrier for conjugation of an alkylating agent to antitumour immunoglobulin (Ig). Drug and primary amine are attached to dextran by cyanogen bromide and the drug-carrier preparation linked to Ig by glutaraldehyde. Conjugates prepared in this way are highly effective in suppressing growth of the EL4 mouse lymphoma. It is demonstrated that the effectiveness of the conjugate is related to the effectiveness of the antiserum from which it is prepared and also that the results are unlikely to be due to drug–antibody synergism. Whether a homing mechanism is involved remains, however, obscure.*

INTRODUCTION

THE USE of polyglutamic acid (PGA) as an intermediate carrier of drug in a drug–antibody conjugate has been described previously [1]. This system was effective in suppressing growth of the EL4 lymphoma in syngeneic mice but proved difficult to control [2] as the degree of coupling drug-carrier conjugate to immunoglobulin was variable. Another variable resulting from the use of PGA as a carrier is the residual net negative charge of the conjugate due to the remaining free carboxyl groups. These groups give water-solubility to the preparation but may result in the conjugate being preferentially taken up by reticuloendothelial cells [3]. The present paper describes the use of an alternative carrier, dextran, which is both neutral and water soluble and which after introduction of amino groups can be coupled to Ig by a standard glutaraldehyde method. Results show that conjugates prepared this way are effective in suppression of tumour growth in mice.

MATERIAL AND METHODS

The alkylating agent PDM (N,N-bis(2-chloroethyl)-*p*-phenylene-diamine) was supplied by the Chemical Defence Establishment, Porton, England or synthesized by Dr. S. Coppell in our own laboratories according to the method of Everett and Ross [4]. Soluble Dextran (M. Wt. 17,700) was obtained from Sigma Chemical Co. Ltd., cyanogen bromide (CNBr.) from Koch-Light Laboratories, U.K. and hexamethylene diamine (HMD) from Hopkin and Williams, U.K.

Normal rabbit globulin (NRG) was prepared from rabbit serum by precipitation with 40% saturated ammonium sulphate twice, redissolving in phosphate buffered saline (PBS) and dialysing against PBS. Rabbit immune-globulin (RIg) was prepared by a similar technique from rabbit antiserum raised against mouse lymphoma cells (EL4), made specific by repeated absorption with normal mouse spleen cells [5].

Preparation of PDM-dextran

One gram of dextran was dissolved in 1 l distilled water and the pH raised to 11·0 by addition of 1 N NaOH. A solution of CNBr (3·3 ml) in acetonitrile at 250 mg/ml was added dropwise to the rapidly stirred dextran at room temperature. The pH was maintained between 10·8 and 11·0 by addition of NaOH. After addition of CNBr, the mixture was stirred with pH maintenance for a further 10 min. HMD (200 mg) dissolved in 5 ml water was then added and the pH lowered to 9·0 with 1 N HCl, and left stirring for 5 min. PDM (500 mg) dissolved in 10 ml ethanolic HCl, plus 40 ml 60% aqueous propylene glycol containing 1·2% w/v K_2HPO4 was then added dropwise, the pH being allowed to fall to 6·5 and there-

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after held at that pH by addition of NaOH. After stirring for 15 min at room temperature the mixture was cooled to 4°C and concentrated to 300 ml by hollow fibre ultrafiltration (Amicon Corp., model DC 2). The same apparatus was used to dialyse with distilled water till the dialysate was free of u.v. absorbing material. The preparation (PDM–DEX) was finally freeze-dried for storage.

Conjugation of PDM–DEX to globulins

Equal volumes of PDM–DEX at 30 mg/ml in water and globulin at 30 mg/ml in PBS were mixed at room temperature and glutaraldehyde gradually added to give a final concentration of 100 μg/ml. After mixing for 1 hr at room temperature the conjugate together with any remaining free globulin was precipitated from the solution by addition of an equal volume of 80% sat. ammonium sulphate. Unconjugated PDM–DEX remained in solution. The precipitate was centrifuged and washed with 40% sat. ammonium sulphate and finally redissolved in PBS. After dialysis the preparations PDM–DEX–NRG or PDM–DEX–RIg were stored at −70°C prior to injection or analysis.

Determination of alkylating activity

Since the conventional method [6] for measuring alkylating activity of drugs using nitrobenzyl pyridine (NBP) involves protein denaturation conditions, any alkylating protein formed by conjugation techniques becomes difficult to quantitate by spectrophotometry due to precipitation. The following modification was devised to retain solubility of the preparation during the assay. A sample of 0·3 ml was added to 0·6 ml acetone: propane-1,2-diol: 0·2 M acetate buffer pH 4·0 (8:16:9 by vol) and 0·15 ml 5% NBP in acetone added. The mixture was incubated at 80°C for 10 min, allowed to cool to room temperature and 0·5 ml acetone: propane 1,2-diol: 1 M potassium carbonate (1:2:3 by vol) added just before measuring absorbance at 600 nm. If the protein concentration of the sample does not exceed 10 mg/ml no loss of solubility occurs and the colour formed due to linked drug can be accurately measured by reference to a standard curve using free drug.

Measurement of in vivo *antitumour effects*

The method was as previously described [7] utilizing groups of mice of C57BL/6 strain inoculated with 5×10^4 live EL4 tumour cells intraperitoneally on day 0. Treatment with conjugates and control preparations was on days 1–4 and effectiveness determined by survival times of the mice.

RESULTS

The *in vivo* effect of dextran-linked PDM conjugates is shown in two separate experiments in Table 1. Different batches of PDM–DEX were used and different antisera served as the source of Ig. The results show that 450 μg PDM (alkylating activity), when conjugated to dextran, had virtually no antitumour effect. The Ig preparation alone (without drugs) increased mouse survival by 12–14 days and by 15 days when injected together with PDM–DEX ("drugged carrier") but unlinked to the Ig. The PDM–DEX–Ig conjugates, however, containing the same amounts of Ig and alkylating activity gave 100% long term survival in one experiment and 60% in the other.

Table 2 shows that the antitumour effect of the alkylating agent is related to potency of the antibody to which it is conjugated. Different immunoglobulin preparations were used ranging in protective antibody content from nil (normal rabbit globulin, NRG) to preparation R210/212 which gave the best protection when used alone.

Although the results shown in Table 1 support the idea that the effect is due to a conjugate and not to an additive or synergistic effect of PDM–DEX and Ig, physico-chemical analysis using gel-filtration has shown that free Ig is present in the materials injected. The possibility therefore must be considered that the preparation is a mixture of PDM–DEX–Ig and free Ig. The antibody activity may be diminished in the conjugate but retained in the free Ig with the result that a mixture could be effective by virtue of a synergism between free Ig and PDM–DEX–Ig. To test this hypothesis, PDM–DEX was conjugated to NRG and a mixture of PDM–DEX–NRG+Ig was compared for effectiveness with a PDM–DEX − Ig preparation. Table 3 shows the result. The conjugate remains the most effective preparation but a synergism between PDM–DEX–NRG and Ig is demonstrable.

DISCUSSION

The problems arising from the direct conjugation to antibody of high levels of hydrophobic anticancer agents have been overcome by the use of intermediate carriers [1]. These provide both multiple drug attachment sites and hydrophilic groups to give the conjugates water solubility. New problems arise, however,

Table 1. *Antitumour effects of PDM conjugates and immunoglobulin*

Treatment*	Dose per injection		Expt. No. RX 95		Expt. No. RX 104	
	Protein (mg)	PDM‡ (μg)	Median survival† (days)	Mice tumour-free at 150 days	Median survival (days)	Mice tumour-free at 150 days
Saline	–	–	13	0/5	14	0/5
Antitumour Ig	6	–	25	1/5	28	0/5
PDM-DEX	–	450	16	0/5	15	0/5
PDM-DEX-Ig (conjugate)	6	450	>150	4/4	>150	3/5
PDM-DEX plus Ig (unlinked)	6	450	28	0/5	29	1/5

*Groups of C57BL/6 mice were challenged with 5×10^4 live EL4 cells i.p. on day 0 and treated on days 1–4 inclusive.

†Median survival is the time to which 50% of the animals in the group survives as defined in Cancer Chemotherapy Reports Vol. 3, No. 2 (1972).

‡As determined using the modified test for alkylating activity described in the text.

Table 2. *Showing that the effectiveness of conjugates is related to the effectiveness of the antisera on which they are based*

Serum batch	% increase in mean survival time relative to control mice injected with saline alone	
	Conjugate treated mice	Globulin alone treated mice†
Normal rabbit serum	7%	0%
R213/218	42%	13%
R219/221	>400%	100%
R210/212	>600%	182%

*The level of drug was approximately 400 μg/injection in each case.

†The globulin was given at the same level as in the appropriate conjugate.

Table 3. *Is the effect due to synergism between free Ig and conjugate?*

Preparation	Median survival (days)	Mice alive at day 150
Saline	14	0/5
PDM-DEX-NRG	15	0/5
PDM-DEX-Ig	150	3/5
PDM-DEX-NRG plus Ig	54	1/5
Ig alone	28	0/5

associated with conjugation of macromolecules; thus preparations contain free drug-carrier conjugates and free immunoglobulin in addition to the drug-carrier-Ig conjugate required. Variability in the preparations was a problem when polyglutamic acid was used as a carrier. The possibility exists of fractionating the preparations by gel filtration but the lability of the alkylating moiety in dilute solutions has made this of little practical value. In the present work, the use of dextran as a carrier has allowed ammonium sulphate precipitation to be used for fractionation. In this way the conjugates can be freed from unlinked PDM-dextran although they still contain free Ig.

The antitumour effectiveness of the preparations can be clearly demonstrated and shown to be an antibody-dependant phenomenon. What has not been proved is that the effect is due to "homing", i.e. selective transport of drug to tumour by specific antibody. The possibility that the observed effects are due to synergism between free Ig and conjugated Ig has been considered. Although the results suggest that this is not the cause, the limited numbers of animals tested in this way do not allow this to be ruled out unequivocally. However, since some loss of antibody activity occurs in conjugation, direct comparison of the two groups involved (PDM–DEX–NRG+Ig versus PDM–DEX–Ig) is not entirely fair since no allowance was made for this loss. This may add more weight to the view that the linked preparation is the most effective and may be working by a "homing" mechanism. Without further experiments, however, the detailed mechanism of action of these preparations must remain supposition.

Since the ultimate aim of the present study is to improve the therapeutic effectiveness of anti-cancer drugs it is valid to consider how the conjugates compare with unconjugated drug. Free PDM was not administered as a

control in the present experiments since when linked to a macromolecule it has totally different toxicological and pharmacological properties. However, we have in previous studies [8] shown that free PDM has very little anti-tumour effect in the EL4 test system and does not result in long-term survivors. Thus the drug-antibody conjugates appear to offer a true improvement in therapeutic index over drug alone.

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Serum Prolactin Concentrations in Benign Breast Disease Throughout the Menstrual Cycle

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Abstract—*Prolactin concentration has been estimated by homologous radioimmunoassay in morning serum samples taken daily throughout the menstrual cycle of normal women and women with benign breast disease. Whereas the median prolactin concentration in* 550 *sera from* 24 *normal cycles was* 0·10 *mu/ml, in* 19 *women with "fibroadenosis" the median of* 500 *values was* 0·15, *and was* 0·27 *mu/ml for* 337 *sera from* 12 *cycles of women who had had breast cysts aspirated. Abnormal daily or weekly prolactin profiles over the monthly cycle were found in benign breast disease, especially in women over* 30 *years old, and most marked in those with cystic disease. A significant positive correlation was present between age and prolactin in cystic disease, but not in the fibroadenosis or normal groups of women. The two benign breast diseases studied here would seem to be clinical conditions susceptible to treatment by prolactin-suppressive agents.*

INTRODUCTION

IT WOULD now appear to be well accepted that prolactin has many varied actions [1] some of which may be concerned in controlling the physiological activity of mammary tissue. Thus growth, interaction with steroid hormones and electrolyte movement are all aspects of prolactin action which justify the study of this hormone in relation to the breast and in particular to breast diseases. The role of prolactin throughout the various stages of the menstrual cycle is however poorly understood, and even the monthly pattern of the hormone's concentration in the blood has given rise to controversy [2]. Although the human breasts undergo cyclical changes in normally menstruating women, it is impossible at present to assess whether the cyclical nature of many benign breast complaints could be related to abnormal prolactin secretions. Furthermore, despite reports of normal concentrations in various benign breast diseases [3–5], these were based on single samples and do not exclude the possibility of abnormal prolactin profiles during some phases of the menstrual cycle.

The investigation now reported was undertaken to establish serum prolactin profiles over the complete menstrual cycle in two clinically different benign breast diseases in women from a wide range of age. Some patients were categorized as having "benign cystic disease of the breast" when breast discomfort or pain led to clinical examination that revealed a palpable lump containing fluid which could be aspirated. The term "fibroadenosis" was used here to denote a diffuse condition of painful, lumpy breasts [6]. These definitions were broad, and diseases of the mammary ducts, including nipple discharge, were excluded, as were solid lumps, whether benign adenomata or malignant cancer. Normal women were studied for comparison.

MATERIAL AND METHODS

Subjects and samples

The subjects were pre-menopausal women with benign disease of the breast. Eleven of them had had one or more cysts aspirated from one or both breasts, and a further 19 had painful lumpy breasts diagnosed clinically as

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having generalised fibroadenosis [6]. The existence of discrete fibroadenomata or microcysts cannot be excluded from the latter group. The range of age for women with cystic disease was 34–48 and for those with fibroadenosis, 24–49 years. These groups were sub-divided to include women in their 20s, 30s and 40s. Normal women aged 22–49 were also studied, but they are considered as one group because no age-related trends in serum prolactin were found in a detailed analysis of these 24 control cycles [7].

None of the subjects had a history of gynaecological disorders nor was taking drugs, hormone preparations or other agents known to affect prolactin, ovarian steroid, or gonadotrophin levels.

Samples of peripheral venous blood (10 ml) were obtained daily, or as often as possible, between 09.00 and 12.00 hr for at least one menstrual cycle. The blood was left to clot, centrifuged, and serum removed for storage at −20°C.

Measurement of prolactin, follicle stimulating hormone (FSH), oestradiol-17β and progesterone

Prolactin was measured by the radioimmunoassay established in these laboratories by Cole and Boyns [8]. Results are given as milliunits/ml M.R.C. Res. Std. A 71/222 where 1 mu = 50 ng prolactin by this assay system. Displacement of 5% of bound iodinated prolactin was achieved by 0·04 mu/ml. Serum samples were assayed in duplicate, all those for an individual subject being together in one of the 8 assays that were required. So far as was possible, each assay contained samples from normal, cystic and fibroadenosis groups, where age and the length of menstrual cycle were comparable for women of each group.

Measurements of FSH were limited to women over 40 yr old. The radioimmunoassay of Groom *et al.* [9] was used and the results served as a check on whether the women had normal cycles with a marked mid-cycle peak.

The results of oestradiol-17β and progesterone radioimmunoassays have been published for these subjects [1, 10].

Analysis of results

Prolactin concentrations were calculated from standard curves using the preferred equation of Taljedal and Wold [11]. Values of less than 0·01 were entered into subsequent calculations as 0·01 mu/ml.

Menstrual cycles varied greatly in length and so two reference points were used: the day of the mid-cycle peak of oestradiol-17β was designated Day 0, taking gonadotrophin and progesterone profiles into consideration; the day of appearance of menstrual bleeding was labelled M.

Serum prolactin concentrations approximated to a log-normal distribution and so Student's *t*-test was applied to mean logarithms for making planned comparisons [12]. Back transformation of mean logarithms yielded the geometric means which have been plotted for simplicity.

The experimental design supported planned comparisons between normal and each of the other groups, and also between benign disease groups within the same age range. Such comparisons would utilize all or similar portions of the prolactin profile over the menstrual cycle. For examining changes occurring within a cycle, the Student–Newman–Keuls procedure was applied as these were essentially unplanned comparisons. Significant effects were not found for any experimental group by this procedure because of inter-subject variation.

Correlation analysis was used for testing the presence of age-related trends of prolactin concentration.

RESULTS

When patients were grouped by diagnosis and age (Table 1), the expected age-distribution was apparent, with cystic disease tending to occur in later years than fibroadenosis. Cycle lengths tended to be more variable than normal for these breast disease patients although the median values were similar. However, the number of days from the mid-cycle oestradiol-17β peak to onset of menstruation was 16·4 ± 2·25 (S.D., 10 cycles) for patients with cystic disease, whereas the fibroadenosis and normal groups had shorter luteal phases (14·4 ± 2·48 and 14·9 ± 1·69 for 16 and 21 cycles respectively: $2P < 0{\cdot}05$ vs cystic group).

In Table 2, prolactin concentrations for each experimental group are given, using three statistics of location with their associated statistics of dispersion. The geometric mean lies closer to the median value than does the arithmetic mean in every case. Furthermore, the range which should contain 95% of the prolactin concentrations around the geometric mean corresponds quite well with the 2·5–97·5 percentiles of the raw data in every group, whereas the standard deviation of the arithmetic mean implies many negative values. Hence a logarithmic transformation was applied to prolactin concentrations, and back-transformation to give geometric means has been

Table 1. *Allocation of subjects to groups, their age and the duration of their menstrual cycle*

Diagnosis	Group	Age (yr)		Cycle length (days)		Number of cycles
		Median	Range	Median	Range	
Normal	—	35	22–49	28	22–32	24
Cystic disease	—	43	34–48	27	22–35	12
Fibroadenosis	—	32	24–49	27	20–39	19
Cystic disease	30s	35	34–37	27	25–29	5
	40s	45	40–48	27	22–35	7
Fibroadenosis	20s	27	24–29	27	20–34	6
	30s	33	31–38	28	26–39	8
	40s	46	40–49	24	20–26	5

Menstrual cycles have been grouped by diagnosis and age. Note that 2 cycles were studied in one 22 yr old normal subject and also in a 35 yr old who had cystic disease.

Table 2. *Comparison of serum prolactin concentrations between groups of subjects using statistics of location and dispersion that are derived from raw data (median with 2·5–97·5 percentiles) and by arithmetic (mean ± S.D.) or logarithmic (geometric mean ± 1·96 S.D. range) computation*

Diagnosis and age group		Number of sera	Median	2·5–97·5 percentiles	Arithmetic		Geometric Mean	± 1·96 S.D. range
					Mean	S.D.		
Normal	—	550	0·10	0·01–0·38	0·13	0·111	0·091	0·01–0·53
Cystic disease	—	337	0·27	0·05–1·16	0·34	0·273	0·252	0·05–1·21
Fibroadenosis	—	500	0·15	0·02–0·88	0·22	0·212	0·154	0·03–0·93
Cystic disease	30s	125	0·17	0·04–1·02	0·24	0·243	0·165	0·03–0·85
	40s	212	0·34	0·07–1·38	0·40	0·273	0·323	0·08–1·27
Fibroadenosis	20s	165	0·13	0·01–0·67	0·17	0·166	0·111	0·02–0·82
	30s	217	0·15	0·04–0·91	0·26	0·239	0·188	0·04–0·95
	40s	118	0·17	0·04–0·91	0·23	0·205	0·172	0·04–0·79

Serum prolactin concentrations in mu/ml MRC Res. Std. A 71/222 are given for menstrual cycles from subjects grouped as in Table 1. The median and 2·5–97·5 percentiles were found by inspection; arithmetic mean and standard deviation (S.D.) were calculated in the usual way; geometric mean ± 1·96 S.D. range are back transformations of the logarithmic mean ± 1·96 S.D. where 1·96 = t (approximately) at the 5% probability level for the appropriate degrees of freedom.

Table 3. *Significance levels by Student's* t*-test for planned comparisons of logarithmically transformed prolactin concentrations sampled over complete menstrual cycles of subjects as in Tables* 1 *and* 2

Diagnosis and age group		Cystic disease		Fibroadenosis		
		30s	40s	20s	30s	40s
Normal	20–50	***	***	*	***	***
Cystic disease	30s	—	***	—	N.S.	—
Fibroadenosis	30s	N.S.	—	***	—	N.S.
Fibroadenosis	40s	—	***	**	N.S.	—

Two-tailed probabilities: ***<0·0005; **<0·005; *<0·02; N.S. >0·16; — not tested.

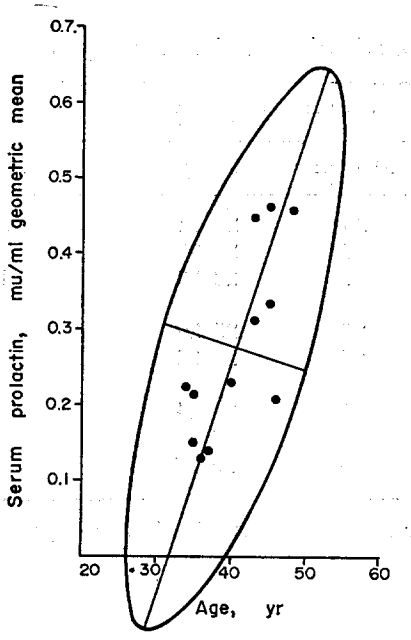

Fig. 1. Relationship between serum prolactin concentration and age in patients with cystic breast disease. The equal frequency ellipse is calculated to enclose 95% of observations of age and the geometric mean prolactin level over the menstrual cycle for this group of women.

adopted for clarity of presentation. Mean logarithms were used for significance testing by Student's *t*-test. As detailed in Table 3, though apparent from Table 2, prolactin concentrations were higher than normal in benign breast disease, especially in women who had had cysts aspirated, and predominantly so in the 40s age group. This group of older cystic patients had higher prolactin levels than either the younger group or the comparable age of fibroadenosis patients. An age-related trend was established for all the cystic patients (Fig. 1) by correlation analysis, which showed that geometric mean prolactin concentration over the complete menstrual cycle was positively correlated with age (Spearman rank correlation coefficient = 0·57, linear correlation coefficient r = 0·749, 10 degrees of freedom). No such correlations were significant for the fibroadenosis group, whether as a whole or as subgroups, nor for the normal subjects.

The daily patterns of geometric mean prolactin concentration are shown in Fig. 2. A striking feature of the benign breast cases is the

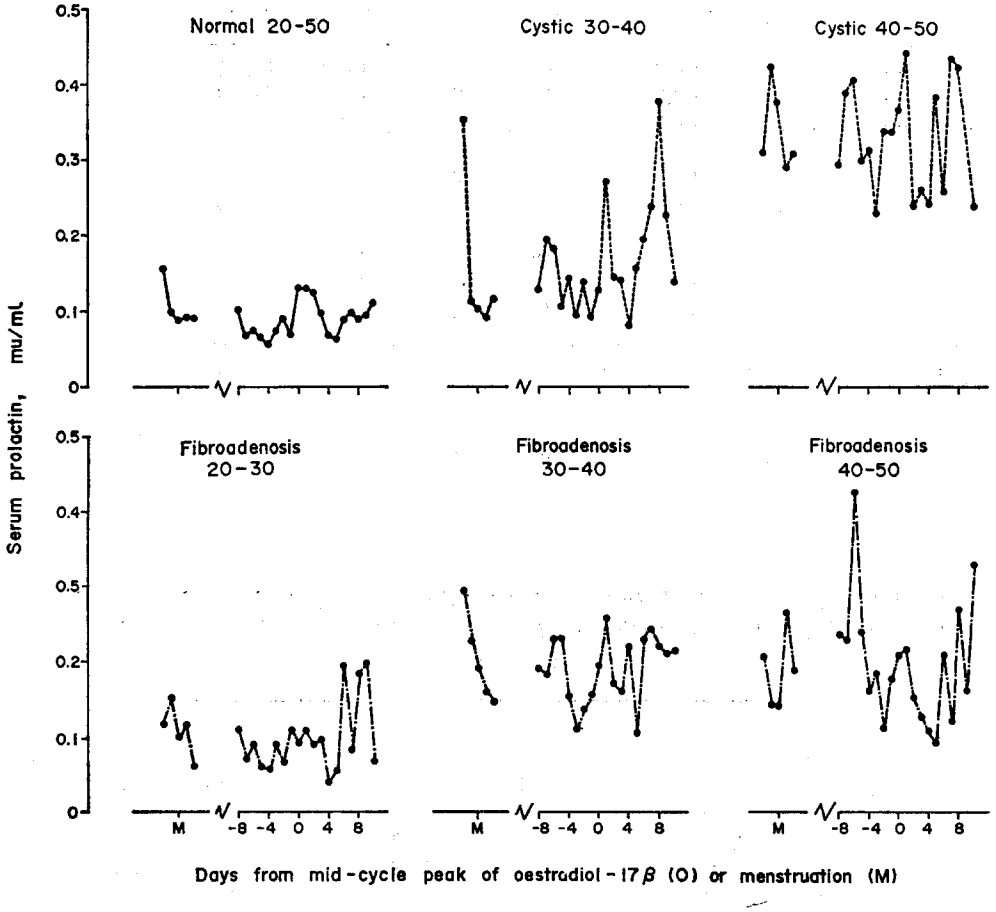

Fig. 2. Serum prolactin profiles of normal women ●——●, and patients with fibroadenosis ●—·—● or benign cystic disease of the breast ●– – –●. Geometric means are shown for days of the cycle aligned by reference to onset of menstrual bleeding (day M) and mid-cycle peak of oestradiol-17β (day 0).

wild fluctuation of serum prolactin level occurring over a few days. The small mid-cycle peak of the normal profile appears pronounced in the cystic breast disease groups and in all but the youngest fibroadenosis group. An abnormal follicular phase peak was similarly present. In the luteal phase, the gradual increase from about day +4 after the mid-cycle oestradiol-17β peak has become a sharp rise of prolactin concentration to a peak at about day +8. This is most clear for the group of women in their 30s in the cystic disease group because all five of them had similar luteal phases which differed by only 2 days.

Weekly patterns for serum prolactin are shown in Fig. 3. Day to day variations and errors due to misalignment of cycle days have been reduced by calculating mean levels over defined 5-day intervals in the menstrual, follicular, periovulatory and luteal phases. A clear pattern is found for normal subjects for whom luteal prolactin concentrations tend to be higher than in the follicular phase, and highest levels occur in the periovulatory phase (Fig. 3). In the benign breast disease patients, however, luteal phase prolactin concentrations were greater than in the periovulatory period, and follicular phase levels were higher than the normal pattern would predict in all but the youngest fibroadenosis group.

Significance levels are given in Table 4 for the planned comparisons that the data of Fig. 3 support. Prolactin concentrations of women with cystic disease were greater than in the normal women in the follicular and luteal phases, and for the remaining weeks in the older group. The cystic 30s group was significantly different from the cystic 40s, except in

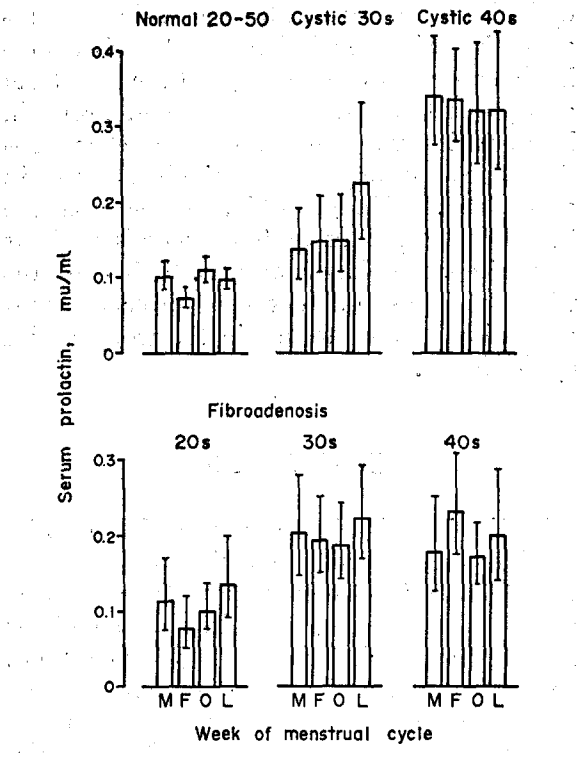

Fig. 3. Weekly patterns of serum prolactin in normal women and in patients with benign breast disease. Geometric means with their 95% confidence limits are shown for results which were pooled over 5-day intervals in the menstrual (M), follicular (F), periovulatory (O) or luteal (L) phases of the menstrual cycle.

Table 4. Significance levels for planned comparisons of prolactin concentrations sampled over 5-day intervals as in Fig. 3

Diagnosis and age group	Phase	Cystic disease		Fibroadenosis		
		30s	40s	20s	30s	40s
Normal 20–50	M	N.S.	***	N.S.	***	***
	F	**	***	N.S.	***	***
	O	N.S.	***	N.S.	***	*
	L	***	***	N.S.	***	***
Cystic 30s	M	—	***	—	N.S.	—
	F	—	***	—	N.S.	—
	O	—	**	—	N.S.	—
	L	—	N.S.	—	N.S.	—
Fibroadenosis 30s	M	N.S.	—	*	—	N.S.
	F	N.S.	—	**	—	N.S.
	O	N.S.	—	**	—	N.S.
	L	N.S.	—	*	—	N.S.
Fibroadenosis 40s	M	—	**	N.S.	N.S.	—
	F	—	*	***	N.S.	—
	O	—	**	*	N.S.	—
	L	—	*	N.S.	N.S.	—

Phases: M, menstrual, days M−2 to M+2; F, Follicular, days −8 to −4; O, periovulatory, days −1 to +3; L, luteal, days +6 to +10. Two-tailed probabilities *** $<0{\cdot}0005$; ** $<0{\cdot}005$; * $<0{\cdot}05$; N.S. $>0{\cdot}05$; — not tested.

the luteal phase, but no significant concentration differences were found when compared to the 30s group of fibroadenosis patients. Yet the older cystic group differed from the same age of fibroadenosis patients at all stages of the cycle. Both the 30s and the 40s group of fibroadenosis patients had significantly raised prolactin concentrations throughout the menstrual cycle, but the youngest group had prolactin levels which were above normal when considered overall and yet non-significantly so in each phase. Table 4 and Fig. 3 suggest that the 30s and 40s groups of fibroadenosis patients have similar prolactin profiles. This contrasts with the gross differences of prolactin concentrations related to age in the women with cystic disease.

DISCUSSION

Earlier investigations from these laboratories [3] indicated plasma prolactin concentrations were similar in benign breast disease, breast cancer at various stages and in control patients admitted to hospital for non-breast conditions. At least 2 other studies [4, 5] have also reported that prolactin levels in benign breast disease were within the normal range. These studies were based however upon single samples taken at random times in the menstrual cycle, which in association with rather imprecise terms like "benign breast disease" or "(poly)cystic mastitis", lessens the apparent discrepancy of previous results with the data now described. Furthermore, only one serum in every four had a prolactin concentration beyond the range found in normal menstrual cycles, even in the cystic 40s group (56/212 sera with more than 0·47 mu/ml). This illustrates the advantage of repeated sampling throughout the menstrual cycle.

The two benign breast diseases which were chosen for study were diagnosed by routine criteria, obtaining histological confirmation if practical. If a patient had a gross cyst from which fluid could be aspirated, then she was placed in the cystic disease group. If breasts had painful lumps, then such a condition was broadly termed fibroadenosis on exclusion of cancer, fibroadenoma, duct ectasia, inflammatory reactions, nipple discharge and many other distinctly defined breast complaints. It is obvious that the fibroadenosis group was much more heterogeneous than the cystic disease group and could obviously include microcysts and sub-clinical forms of other breast conditions.

Both cystic disease and fibroadenosis tend to become more severe in the few days before menstruation, and the symptoms disappear after the menopause [13] thus relating benign breast disease to ovarian function. As illustrated in Fig. 2, serum prolactin does not appear to follow oestrogens in a well defined monthly cycle [5, 7, 14–17], although it would seem that oestrogens play an important role in controlling secretion of human prolactin, and Vekemans and Robyn [17] have found a significant decline of prolactin concentration at the menopause in normal women blood donors. In a case of premenstrual syndrome associated with thirst and water retention, prolactin-suppressive therapy with bromocriptine has successfully relieved all symptoms for over a year [18]. Therefore, a cyclical variation of clinical symptoms may be linked to changes in serum prolactin concentration.

In the cystic disease group, there was a clear correlation between prolactin and age that was not present in the other groups. This correlation was in the opposite direction to that found by Vekemans and Robyn in their 86 normal women over their wider age range of 18–65 yr. Although cystic disease and raised prolactin may be associated, this does not prove that one causes the other nor does it exclude a common causative factor. However, the well established role of prolactin in water and electrolyte balance [1] has counterparts in humans as shown by the lowering of serum prolactin by water loading [19] or the relief of water retention by suppression of prolactin secretion [18]. Furthermore, a correlation has been demonstrated between the mean plasma prolactin concentration of male subjects and urinary Na/K excretion [20, 21]. The sign of this correlation became negative when diuretics were administered, which suggested that the diuretic response of an individual was a function of his prolactin set-point. As a working hypothesis then, serum prolactin concentration could control the movement of water and electrolytes into microcysts. A gross cyst would be formed as a consequence of localized fluid influx, and a more generalized swelling of microcysts may account for the cyclical pain in fibroadenosis. Normal breasts, which may also have microcysts [13], are in a prolactin environment that does not favour cyst enlargement.

The incidence of breast cancer in patients with gross cystic disease has been found to be 4 times higher than expected [13], but the raised prolactin levels in cystic disease cannot be given a role in carcinogenesis on the present

evidence since extrapolation is not possible from the 11 women of this study to the 1693 cases of cystic disease in the other study which included 72 that developed cancer [13]. Indeed, patients that have had mastectomy for breast cancer were found to have only minor abnormalities in their serum prolactin profiles throughout the menstrual cycle [7].

If the generally raised serum prolactin concentrations of these two benign breast conditions is a disease factor, then prolactin suppressive therapy would be effective. Such treatment is presently undergoing clinical trials which will be reported at a later date.

Acknowledgements—We thank the Tenovus Organisation, the Medical Research Council and the Cancer Research Campaign for generous financial support.

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A Differential Interaction *In Vitro* of Mouse Macrophages with Normal Lymphocytes and Malignant Lymphoma Cells

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Abstract—In vitro *studies have shown that non-activated mouse peritoneal macrophages establish a differential interaction with mouse normal and malignant lymphocytes from syngeneic animals. The number of malignant lymphoma cells (YAC) bound to macrophages after 60 min of incubation at 37°C is 3–5 fold higher than that of normal lymphocytes. The binding of lymphoma cells to macrophages persists for at least 72 hr after cell–cell contact formation, whereas the normal lymphocytes detach from macrophages during the first 24 hr of incubation. Incubation of macrophage-lymphoma cell rosettes for 72 hr results in an extensive phagocytosis of about 50% of the bound lymphoma cells. However, replacement of the culture medium with fresh medium every 24 hr bolished the ingestion of the lymphoma cells without changing their attachment to rop ahages.*

INTRODUCTION

DIVERSE functions have been ascribed to mononuclear phagocytes. Among the most extensively studied are those involving phagocytosis of invading organisms and senescent self cells, killing of tumour cells, interaction with immunocompetent lymphocytes, elimination of cell debris in inflammation and secretion of various macromolecules [1–4]. There is no doubt that expression of several of these functions demands an intimate contact between the macrophages and the cell on which the function is exerted. Evidence is accumulating, however, of the release of soluble factors from macrophages, either under normal conditions or due to external nonspecific or immunologic stimulation that may replace the macrophage [5, 6].

A wide range of reports on macrophage cytotoxicity seems to establish contradictory concepts. According to the different experimental designs, cytotoxicity was found to be both non specific in some systems [7–10] and specific in others [11, 12]; killing was observed in some [9, 10, 13] whereas in others macrophages brought about cytostatic effects [7, 8, 14]. While several authors report on spontaneous nonspecific cytotoxicity towards tumour cells in "normal" macrophages derived from unstimulated animals [8, 15]. others report on development of such activities only in macrophages infected with bacteria and parasites [10, 16, 17]. Immunologically specific macrophage cytotoxicity depending on T-cell arming factors has also been extensively studied [18, 19] An unexpected observation of Nathan and Terry [20] adds to the already confused situation in that macrophages were shown to exert a differential stimulation of murine lymphoma cells.

Close contacts between guinea pig macrophages and lymphocytes have been shown to develop via an immunological species and strain specific mechanism as well as via a non immunological species but not strain specific mechanism [21–23], Lymphoma cell growth *in vitro* was shown to be inhibited by normal macrophages [15] and stable cell–cell contact between lymphoma cells and normal macrophages have also been described [24]. In both cases specific conditions could be found where immunological activated macrophages were more efficient.

The following study was undertaken to determine (a) whether there is a differential attachment of normal and malignant lympho-

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cytes to mouse peritoneal macrophages and (b) whether the attachment of the two cell types to macrophages is followed by phagocytic events.

MATERIAL AND METHODS

Media

Sterile phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (EM) supplemented with 100 u/l penicillin and 100 μg/l streptomycin and heat inactivated newborn calf serum (NCS) were obtained from Grand Island Biological Co. (N.Y.).

Cells

Peritoneal macrophages were aseptically collected from A and BALB/c strain mice (4–6 weeks old), following essentially the method of Cohn and Benson [25]. Peritoneal exudate cells suspended in medium were allowed to attach (1 hr, 37°C) on 25 mm diameter Corning cover-glasses, placed in Flacon plastic tissue culture dishes (35 × 10 mm, Falcon Plastics Div. Bioquest, Oxnard, Calif.). Peritoneal exudate cells containing about $0{\cdot}5 \times 10^6$ macrophages were applied in 0·2 ml medium to the cover-glasses. The attached cell monolayers were thoroughly rinsed with PBS in order to remove the nonadhering cells. The cultures were then cultivated for 24 hr at 37°C in 2 ml of 20% serum in medium, in a CO_2-incubator (5% CO_2–air mixture).

Thymus derived lymphocytes, splenocytes and lymph node cells of A mice were collected by teasing the respective organs apart. Tissue pieces and visible cell clumps that settle to the bottom of the tube by gravity were discarded.

Malignant lymphoma cells, an ascites form of a Moloney virus-induced lymphoma (YAC) [26] were grown in A strain mice by intraperitoneal inoculation of 10^5 cells per animal, and collected for experiments 11–14 days after inoculation.

For the experiments normal lymphocyte and malignant lymphoma cells were freshly collected from animals in PBS, washed twice and resuspended in medium before use.

Interaction of macrophages with normal and lymphoma cells

Macrophage monolayers were washed twice with PBS and exposed to 30×10^6 normal lymphocytes or lymphoma cells in 2 ml of medium for 60 min at 37°C. Culture plates were subsequently washed twice in PBS and were either fixed (2% glutaraldehyde 30 min, 4°C) or reincubated in 20% serum in medium for 72 hr and fixed. Culture plates were then stained (Giemsa) and cells attached per 100 macrophages were enumerated in tetraplicate cultures. Attached cells were readily distinguished from ingested cells by the colour changes and irregular contour of the latter. The stained cultures were examined and photographed with Karl Zeiss Ultraphot microscope.

RESULTS

Attachment of normal lymphocytes and malignant lymphoma cells to macrophages Thymus derived lymphocytes establish few stable cell–cell contacts with the macrophages (Fig. 1b) whereas lymphoma cells form stable rosettes at the periphery of the macrophages (Fig. 1c). Quantitation of the differential interaction of thymus derived lymphocytes and lymphoma cells with macrophages established a 3–5 fold higher interaction for the latter (Table 1). Macrophages are free of any interaction with extraneous cells unless incubated with thymus derived lymphocytes or lymphoma cells (Fig. 1a). Binding of YAC lymphoma cells to macrophages was not affected even by *in vitro* growing of the lymphoma cells for two passages.

Table 1. *Malignant lymphoma cell and normal lymphocyte attachment to macrophages**

Exp. No.	Lymphoma cells attached/100 macrophages ± S.E.	Normal lymphocytes attached/100 macrophages ± S.E.
1	256 ± 11·2	68 ± 7·6
2	220 ± 14·8	48 ± 4·0
3	292 ± 22·8	80 ± 9·0

*Macrophages were incubated with 30×10^6 lymphoma cells or thymus-derived normal lymphocytes in medium for 60 min at 37°C.

Macrophage–lymphocyte rosette formation following macrophage incubation with either lymph node lymphocytes or spleen cells was of the same extent as that established with thymus-derived lymphocytes.

Analysis of the distribution curve of lymphoma cells attached to macrophages indicate that 88% of macrophages establish stable contacts with lymphoma cells and the number of attached cells per macrophage appears to follow a Gaussian distribution. Thus the whole macrophage population is apparently capable

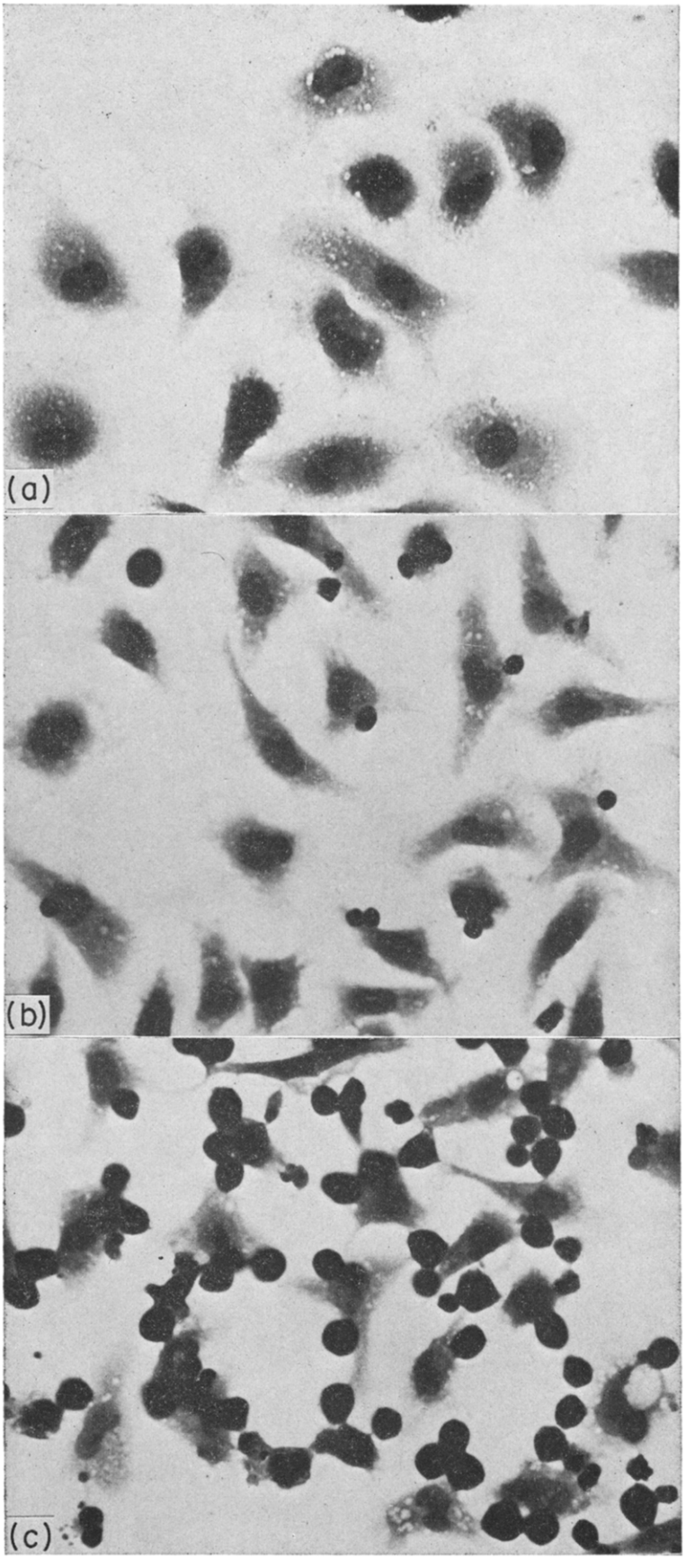

Fig. 1. *Physical interaction of macrophages with thymus derived lymphocytes and malignant lymphoma cells. Macrophages were incubated for* 60 *min at* 37°*C with either* (*a*)*-medium,* (*b*)*-thymocytes and* (*c*)*-lymphoma cells at a concentration of* 30×10^6 *cells/ml.* × 850.

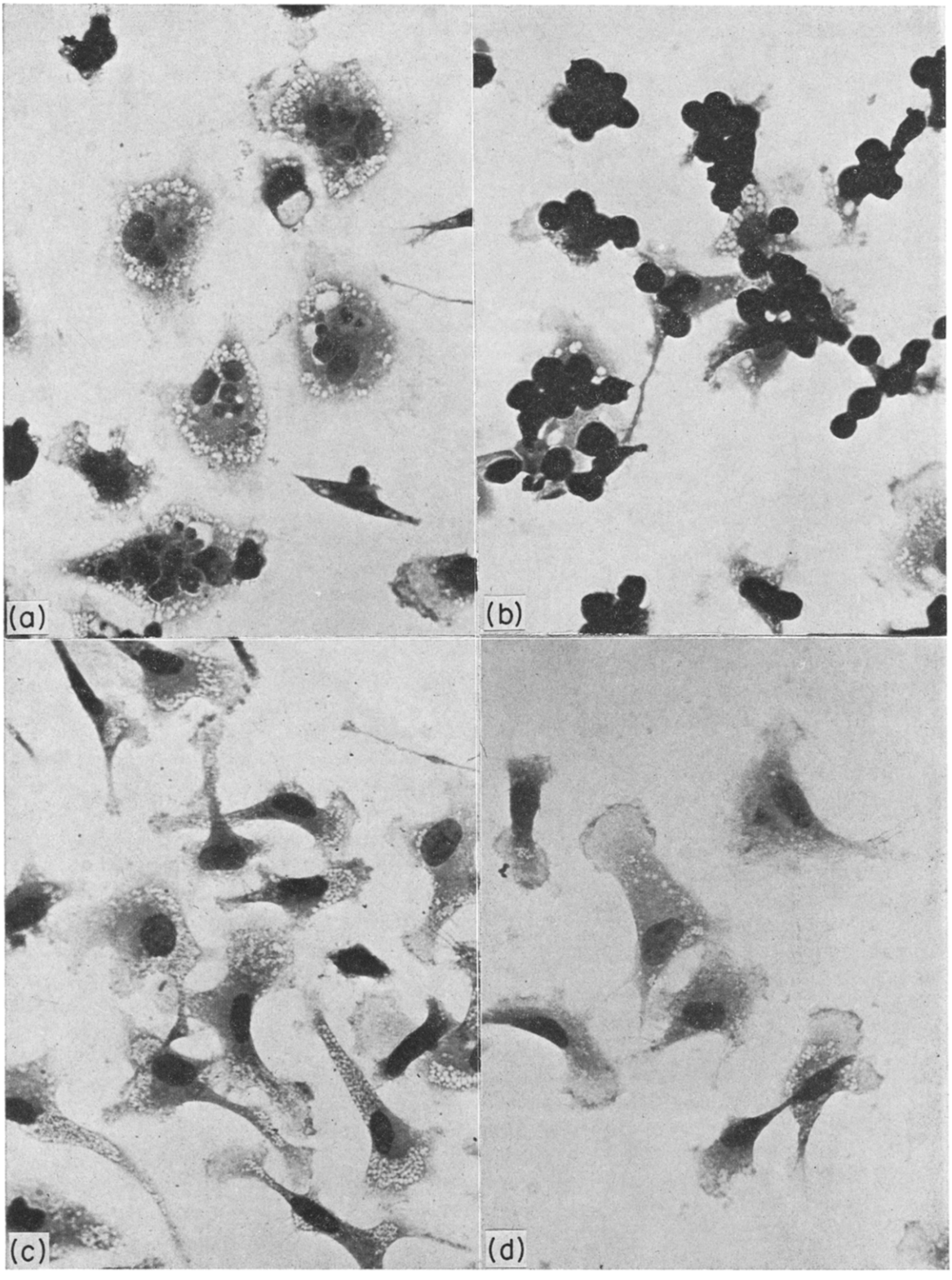

Fig. 3. *Lymphoma cell ingestion by macrophages. Preformed rosettes of macrophage lymphoma cells* (*see Fig.* 1*c*) *were cultured for* 72 *h* (*a*)—*without culture medium replacement and* (*b*)—*with culture medium replacement every* 24 *h.* (*c*) *and* (*d*)—*macrophages that were not challenged with lymphoma cells cultured under conditions of* (*a*) *and* (*b*), *respectively.* × 850.

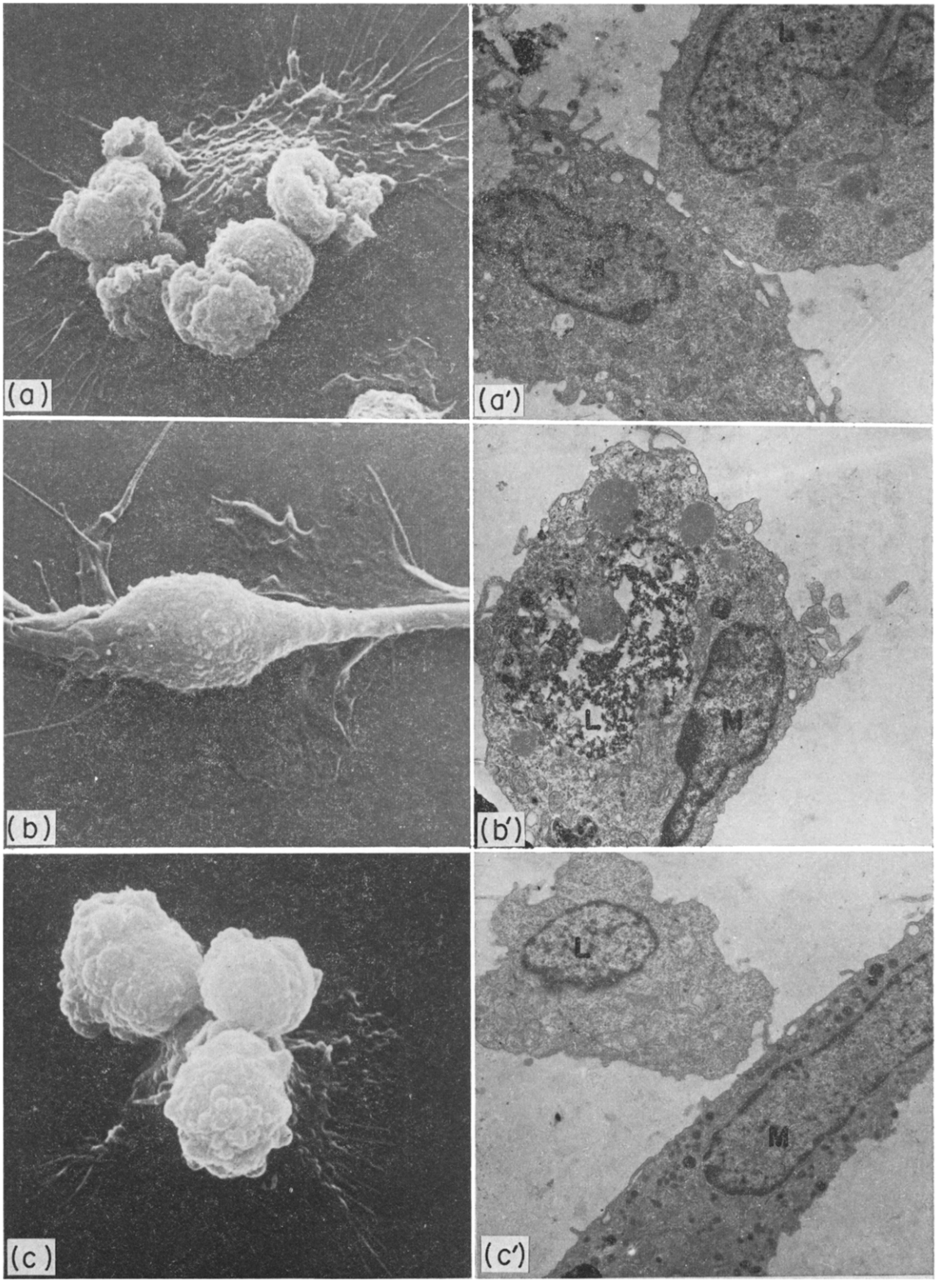

Fig. 4. Electron microscopy of YAC lymphoma cell-macrophage interactions. Macrophages YAC lymphoma cells after 60 *min of interaction at* 37°C (*a* and *a'*); *ingestion of lymphoma cells by macrophages* 72*hr after incubation* (*b and b'*); *ingestion of YAC lymphoma cells abolished by culture medium replacement*(*c and c'*), (*Y-YAC lymphoma cells; M-macrophages*).

Scanning electron microscopy cells left grown on 13 *mm corning cover glasses were fixed with* 2% *glutaraldehyde in* 0·1*M Na-cacodylate buffer* (*pH* 7·4) *for* 1 *hr at* 24°C. *After washing three times in the buffer, dehydration in increasing concentrations of ethanol was followed by acetone. The preparations were dried with a critical point drying apparatus* (*Polaron Equipment Ltd., Watford, Herts*). *The dried specimens were coated with gold using rotating stage vacuum evaporator. A steroscan Cambridge S-*180 *scanning electron microscope was used at an acceleration voltage of* 30 *kV and tilt angle of* 30°. × 1900.

Electron microscopy cells (*right*) *grown on parlodin sheets were prepared for visualization by electron microscopy according to Spurr* [39] *as described by Raz and Goldman* [40]. *Thin sectioning was obtained with Sorvell, Porter–Blum MT-*2 *ultramicrotone and analyzed in a Philips EM-*300 *electron microscope operated at* 80 *kV.* × 6300.

of forming cell–cell connections and the adherence phenomenon is not limited to a small subclass of macrophages (Fig. 2). A small subclass of macrophages incapable of binding of lymphoma cells is however not excluded.

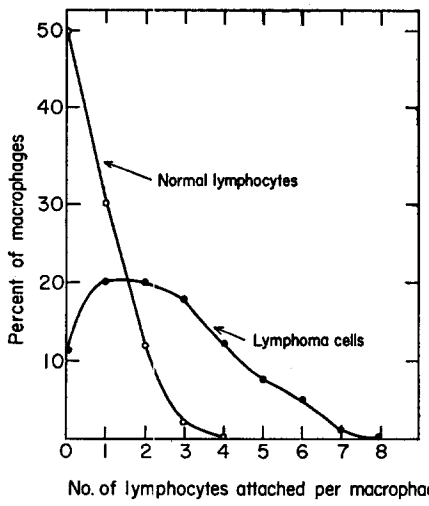

Fig. 2. Distribution curves of thymocyte and lymphoma cell attachment to macrophages. Based on experiments 1–3 *in Table* 1.

To assess whether the macrophage–lymphoma cell association is strain dependent, macrophages derived from BALB/c mice were cultivated and interacted with lymphoma cells under conditions comparable to the above. BALB/c macrophages were found to establish 10% of the cell–cell association observed with macrophages derived from A mice.

Ingestion of malignant lymphoma cells by macrophages

Most of the studies reporting nonimmunological cytotoxic effects of nonspecifically activated peritoneal macrophages on malignant target cells were carried out under conditions of prolonged incubation of the interacting cells. Destruction of target cells is readily observable at 60–72 hr of cell–cell interaction and does not involve phagocytic events though cell debris has been shown to be disposed of within the macrophages [24].

Incubation of lymphoma cell–macrophage rosettes for 72 hr in 20% serum–medium results in up to 50% interiorization of the attached lymphoma cells (Table 2). The total number of lymphoma cells associated with macrophages (ingested — attached) at 72 hr incubation is equivalent to the number associated with macrophages at the beginning of the incubation, namely after a 60 min incubation with lymphome cells and ×2 wash in PBS. It is worth mentioning at this point that thymus derived lymphocytes detach from the macrophages in the course of the first 24 hr of incubation and therefore no comparable studies with these cells could be carried out.

The light micrographs of macrophage–lymphoma cells cultured for 72 hr (Fig. 3a) suggest that normal macrophages are capable of both ingestion and digestion of lymphoma cells. The morphological observations further indicate that whole lymphoma cells are ingested and not just fragments of debris derived from degenerating cells. Instead of the strong homogeneous staining pattern of attached lymphoma cells, those ingested stain by Giemsa in a faded colour and non-homogeneously. Macrophage–lymphoma cell rosettes incubated for 24 hr show a very low index of phagocytosis (about 5% of attached lymphoma cells are ingested). Particles or cells that are phagocytozible by macrophages are usually ingested within a period of minutes of the time of their attachment.

Macrophage maturation in culture involves an increase in phagocytic capacity [27]. On the other hand continuous secretion of biologically active macromolecules including various enzymes from macrophages as well as from the lymphoma cells may modulate both the macrophage and the attached lymphoma cell plasma membrane and metabolism. In order to establish whether the maturation of macrophages *per se*, the aging of lymphoma cells or components accumulated in the culture medium within the 72 hr are detrimental in the delayed phagocytic response (most effective only after 48 hr incubation of rosettes) the following experimental procedure was adopted macrophage–lymphoma cell rosettes were incubated for 72 hr (37°C) in 20% serum–medium as above but culture medium was replaced by a fresh medium at 24 hr intervals. A striking difference in the fate of the lymphoma cells in indicated in Fig. 3(b). Practically no ingestion was detected under this condition, the cells remain adherent to macrophages and seem to form clusters over the entire area of the macrophage. Additional evidence to support the results obtained with the light microscopy techniques (Fig. 1, 3) was gathered by the aid of scanning and transmission electron microscopy (Fig. 4).

The differential interaction of macrophage–lymphoma cells with and without serum–medium replacement excludes the maturation of macrophages as the detrimental factor for the observed phagocytosis at 72 hr as opposed to lack of phagocytic activity at 24 hr. It should

be emphasized however that macrophages cultured for 72 hr with and without serum–medium replacement may differ to some extent in their metabolic activity and endocytic activities [27]. Figs 3c and 3d indicate a morphological difference under the two culturing conditions expressed in abundance of minute phase lucent vesicles in cultures incubated in serum–medium for 72 hr without replacement as compared to moderate vesicle formation in cells incubated with fresh serum–medium every 24 hr.

DISCUSSION

Normal macrophages have been shown to establish a differential interaction pattern with lymphocytes and malignant lymphoma cells. The interaction with lymphoma cells involves both a high degree of rosette formation (Table 1) and the development of stable cell–cell contacts; i.e., the number of attached lymphoma cells is not reduced with a 72 hr incubation period (Fig. 3b). The degree of rosette formation with normal lymphocytes derived from the thymus, spleen and lymph nodes is several times lower than that with lymphoma cells and the attached lymphocytes detach within the first 24 hr of incubation.

Viral induction of malignant transformation, changes various parameters of cell physiology. One of the most extensively studied parameters is that involving changes in plasma membrane composition, organization and function [28–29].

The high degree of macrophage interaction with lymphoma cells reported in the above could reflect a recognition by the macrophage of alterations in surface properties of the attached cell. It is of interest that this interaction depends on syngeneity of macrophages and lymphoma cells; namely macrophages from BALB/c mice that do not serve as hosts for YAC lymphoma cell transplantation do not form extensive interactions with the lymphoma cells. Activated mouse macrophages exhibit a cytotoxic effect on tumorigenic 3T12 and SV 40 3T3 transformed fibroblasts and are noncytotoxic to their nontransformed parental cell-line 3T3 fibroblasts [9]. Lipsky and Rosenthal [21] report however that two normal lymphoid cell populations, thymus and lymph node lymphocytes, were bound to syngeneic guinea pig macrophages in significantly larger numbers than L_2C leukemia cells. In the guinea pig macrophage–thymocyte interaction is exceptionally high, amounting to up to 300 thymocytes per 100 macrophages [21, 22, 30]. In the A mouse our results indicate a much lower degree of interaction of thymus-derived lymphocytes with macrophages.

Stable cell–cell associations of lymphocytes and macrophages have been frequently observed during *in vitro* cultivation of these cells [31–33], as well as in fixed preparations of lymphoid organs [34, 35]. Under *in vivo* conditions clusters of lymphocytes and blast cells around macrophages are formed in response to immunization procedures [34, 35]. Likewise the *in vitro* induction of primary antibody responses and antigen-mediated *in vitro* proliferation of immune lymphocytes [32, 33] has been shown to involve direct physical contact between lymphocytes and antigen-containing macrophages. Lymphocytes have also been found to adhere to macrophages both *in vivo* and *in vitro* in the absence of specific immunization [21, 22]. When macrophage–lymphocyte associations involve antigen [2] or lectin [6] presentation, the result of the association is lymphocyte proliferation. Very rarely have phagocytic events been observed.

The association of nonimmune and nonactivated peritoneal macrophages with a variety of lymphoma cells has been reported to result in inhibition of proliferation and (^{3}H)-thymidine uptake [15]. Many of the studies suggest the need for macrophage–lymphoma contact and indicate that though immune and nonspecifically activated macrophages are more efficient in their growth inhibition capacity, normal macrophages are also endowed with cytostatic as well as cytotoxic capacity [14, 24].

Most experimental systems studied use macrophage-target cell ratios of 100:1, 40:1, 10:1, 5:1 and rarely a ratio of 1:1 [7, 14, 17, 24]. The differential activity of activated vs normal macrophages is mostly evident under conditions of a low ratio of macrophage to target cell [14, 24].

In our studies we have used a ratio of 1:60 of macrophages to target cells. This ratio enabled the establishment of extensive interactions of macrophages and lymphoma cells; on the average 2.5 lymphoma cells were associated with each macrophage in the culture.

An incubation of macrophage–lymphoma cell rosettes for 72 hr without culture medium replacement results in an extensive phagocytosis of adherent lymphoma cells (Table 2). Up to 50% of macrophages were involved in phagocytosis of lymphoma cells and up to 50% of the adherent cells were ingested. In contra-distinction when culture medium was changed every 24 hr for fresh culture medium the macrophage adherent lymphoma cells preserved their stain-

Table 2. Ingestion of lymphoma cells attached to macrophages*

Exp. No.	Percentage of macrophages with at least one ingested lymphoma cell ± S.E.	No. of ingested lymphoma cells/ 100 macrophages ± S.E.	No. of attached lymphoma cells/ 100 macrophages ± S.E.	Total No. of macrophage associated lymphoma cells ± S.E.
1	50 ± 3	116 ± 12	121 ± 14	237 ± 17
2	48 ± 2	108 ± 10	158 ± 22	266 ± 24
3	36 ± 2	44 ± 3	218 ± 4	262 ± 16

*Preformed rosettes of macrophages and lymphoma cells (for conditions and quantitation see Exp. 1–3 in Table 1, respectively) were incubated for 72 hr in serum-medium at 37°C.

ing characteristics with the Giemsa stain, and were all attached to macrophage surface.

Extensive ingestion of lymphoma cells has not been reported hitherto though Lejeune and Evans have observed engulfment by macrophages of lymphoma debris [24]. An early report by Bennett *et al.* [36] suggests that even peritoneal macrophages from immunized mice do not exhibit an inherent specific ability to phagocytose tumour cells *in vitro*, unless the medium contained a humoral antibody against the tumor.

The differential interaction of macrophage–lymphoma cells under conditions of culture medium replacement and continuous culture in the same medium is not fully understood. Several possible processes may be involved in the phenomenon, either of which can be the major responsible factor or they may exert synergistic effects.

During the extended incubation time of macrophage–lymphoma cell rosettes both cell types may undergo metabolic as well as surface changes. Recent studies in our laboratory indicate a continuous maturation process of macrophages in culture, during which the phagocytic activity increases and surface morphology changes [27]. The functional changes during maturation depend on whether or not the culture was incubated continuously in the same serum medium or whether culture medium was replaced every 24 hr. Figures 3(c) and 3(d) amply show the differential morphology of macrophages under the above-mentioned conditions. Moreover, macrophages release factors and various enyzmes to the medium [1, 37]. Their accumulation within 72 hr of incubation may act on both the macrophages as well as on the adherent lymphocytes and render them more susceptible to phagocytosis. Metabolic changes, cytostatic or cytotoxic effectors may serve as signals for macrophage scavenging capacity. Bennett *et al.* [36] have in fact shown that under certain conditions killed tumor cells are readily phagocytosed even without opsonization with isoantibody. If mediated by products released from macrophages, the fact that almost no phagocytosis is observed under conditions of culture–medium replacement means that a concentration factor for the product has to be involved. Either its secretion within 24 hr is not sufficient for triggering phagocytic events or the enriched aged medium enhances in itself product secretion. Macrophage challenge with tumor cells has been shown to cause rapid changes in lysosomal morphology and content [24]. It is not impossible that rosette formation serves in itself as a challenge for macrophage activation. That this would be more effective without culture medium replacement can stem from need of factors released by either macrophages, that upon activation release more enyzmes and biologically active products [28], or by lymphocytes. So that in addition to macrophage involvement in the phagocytic event, lymphocytes could mediate potentiation of macrophage function.

The various aspects of *in vitro* lymphoma cell interaction with macrophages and the phagocytic events that conclude it are currently under investigation. It is hoped that studies of the *in vitro* macrophage–lymphoma cell interaction would help to understand the *in vivo* situation where 10^3 lymphoma cells injected i.p. escape *in vivo* the resident peritoneal macrophage population ($1–2 \times 10^6$ cells), proliferate and kill the mouse within 20–25 days.

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Protocol for a Cooperative Trial of Empirical Antibiotic Treatment and Early Granulocyte Transfusions in Febrile Neutropenic Patients

E.O.R.T.C.* INTERNATIONAL ANTIMICROBIAL THERAPY PROJECT GROUP†

**E.O.R.T.C.: European Organisation for Research on Treatment of Cancer*

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INTRODUCTION

THE RECENTLY completed E.O.R.T.C. trial [1] indicates that (a) empirical treatment with carbenicillin + gentamicin, carbenicillin + cephalothin and cephalothin + gentamicin cures respectively 69%, 45% and 62% of gram negative bacteremias in neutropenic patients; (b) significant nephrotoxicity occurs in 15% of the patients treated with cephalothin and gentamicin compared to those treated with cephalothin + carbenicillin (6%) and carbenicillin + gentamicin (2·4%).

Thus the "optimum" empirical antimicrobial regimen at present available (carbenicillin + gentamicin) still leaves ample room for improvement. This improvement may be obtained by adding to the carbenicillin + gentamicin combination a third drug (a cephalosporin) and/or by giving to the infected patient, early in the course of his infection, transfusions of granulocytes; the exact value of this treatment for infected neutropenic patients has still to be defined. It would be best however, for transfusions of granulocytes to be tested in a "poor risk" group of patients, defined by an analysis of the data from the last trial.

The "poor risk" group of patients will consist basically of those patients who are predicted to have gram negative septicemia and whose bone marrow is not expected to show any sign of recovery within 7 days following the start of therapy. The following are predictive of gram negative septicemia: (1) granulocyte count < 100/μl; (2) creatinine level > 1·0 mg%; (3) temperature > 39·0°C; (4) platelet count < 50,000/μl. All the patients eligible on these criteria for granulocyte transfusion will undergo a bone marrow aspiration (bone marrow aspirations performed between 48 hr before and 24 hr after onset of therapy are acceptable); retrospectively only patients with a hypocellular marrow (0 or + on a scale of 0 to + + + + where + + + is normal, as used by ALGB) will be considered to belong to the "poor risk group" provided they also present 3 out of 4 factors predictive of gram negative bacteremia mentioned above. Patients who are started on the protocol and who, retrospectively, do not have a hypocellular marrow will be considered as "protocol violations".

The patients should be followed in the protocol until discharge from the hospital or death.

Amikacin will be substituted for gentamicin because of its twice daily dosage and better *in vitro* activity against Klebsiella. Cefazolin will replace cephalothin as cephalosporin.

The study is designed to answer the following questions:

(1) Does the addition of cefazolin to amikacin + carbenicillin improve the response of infections in neutropenic patients? (Poor and good prognosis patients will be studied separately).

(2) Does a moderate dosage of cefazolin added to amikacin + carbenicillin carry a

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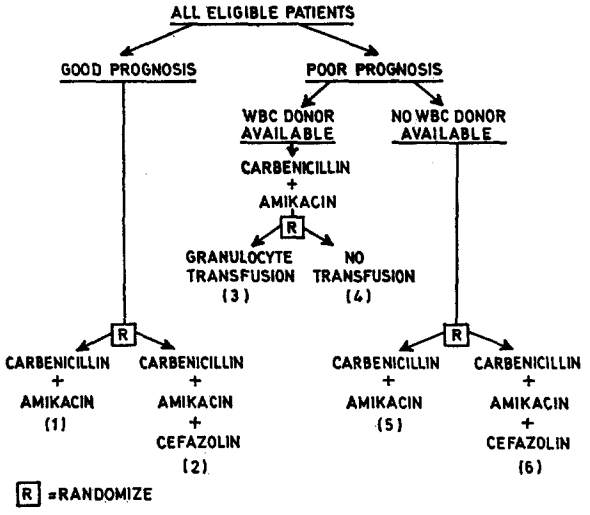

Fig. 1.

risk of nephrotoxicity similar to that found in the previous E.O.R.T.C. study?

(3) In poor-risk patients what is the value of early administered granulocyte transfusions?

(4) Do differently collected granulocytes affect the outcome of severe infection in neutropenic patients in a different way?

(5) What are the side effects of transfusions of granulocytes and can they be related to the mode of collection of the granulocytes and to other factors such as premedication of the donor and histocompatibility?

(6) What are the causes of death in infected neutropenic patients?

(7) Can gram negative bacteremia and the implied subsequent poor prognosis be predicted by the criteria given above?

E.O.R.T.C. INTERNATIONAL ANTIMICROBIAL THERAPY PROJECT GROUP
EMPIRICAL ANTIBIOTIC/GRANULOCYTE TRIAL

RANDOMIZATION CARD

PATIENT NAME: _____ PATIENT-TRIAL NO: _____
HOSPITAL: _____ HOSPITAL NO: _____
INVESTIGATOR: _____ PATIENT AGE (YRS): _____
RANDOMIZED BY: _____ DATE OF RANDOMIZATION: _____

a) GRANULOCYTE COUNT: If < 100/μℓ enter 1; otherwise 0 ___
b) SERUM CREATININE: If > 1.0 mg % (88.4 mmol/L) enter 1, otherwise 0 ___
c) TEMPERATURE: If > 39.0°C (102.2°F) enter 1; otherwise 0. ___
d) PLATELET COUNT: If < 50,000/μℓ enter 1; otherwise 0. ___

TOTAL ___

If the TOTAL score = 0, 1 or 2 go to Section A (Good prognosis)
If the TOTAL score = 3 or 4 go to Section B (Poor prognosis)

A. GOOD PROGNOSIS PATIENTS
Allocated regimen is:-
1. CARBENICILLIN + AMIKACIN
2. CARBENICILLIN + AMIKACIN + CEFAZOLIN
GO TO SECTION E

B. POOR PROGNOSIS PATIENTS
Is there a granulocyte donor available? YES ___; NO ___
If YES go to Section C. If NO go to Section D

C. POOR PROGNOSIS, DONOR AVAILABLE
Allocated regimen is:-
3. CARBENICILLIN + AMIKACIN + GRANULOCYTES
4. CARBENICILLIN + AMIKACIN (NO GRANULOCYTES)
GO TO SECTION E

D. POOR PROGNOSIS, NO DONOR
Allocated regimen is:-
5. CARBENICILLIN + AMIKACIN
6. CARBENICILLIN + AMIKACIN + CEFAZOLIN
GO TO SECTION E

E. ALL PATIENTS: Enter number (1-6) of allocated regimen

Please complete and return this randomization card to the Statistical Center (Dr. H. Gaya) IMMEDIATELY
Retain the Clinical Report Form. Complete and return it to the Statistical Center within 4 weeks.

Fig. 2.

PROTOCOL (FIG. 1)

(A) *Eligibility*

Patients with neutropenia (< 1000 neutrophils/μl) and with fever (> 38·5°C) in the absence of obvious non-infective causes.

Patients are allocated to their treatment by drawing the next envelope from the stock supplied to each participating center. The required patient details must be entered immediately on the postcard provided (Fig. 2) and sent to the Statistical Center, even if the patient is subsequently excluded from the trial. Patients with known severe allergy to any of the anti-biotics in any of the regimens are excluded. Patients with any serum creatinine level are eligible; however, in patients with increased creatinine levels an adjustment of the dosage of amikacin should be made. The method proposed by Cutler and Orme (*J. Am. Med. Ass.* **209,** 539, 1969) can be used here: interval between 2 consecutive doses (in hours) = creatinine level in mg% × 9.

Patients may be included in the protocol more than once, provided there is an interval of 20 days between the two onsets of therapy and provided the patient has been off antimicrobial therapy for 5 days prior to the onset of the second treatment.

(B) *Investigations*

(a) *Before starting treatment:* (1) *Bacteriological.* Blood cultures (3 ×), swabs from relevant lesions, urine and sputum and pharyngeal swab or transtracheal aspirated material if feasible. All significant bacterial pathogens are tested by the Kirby–Bauer technique for susceptibility to carbenicillin (100 μg discs), cefazolin (30 μg discs) and amikacin (10 μg discs). Determination of the minimal inhibitory concentration is desirable but optional. (Use 10^5/ml inoculum and Mueller–Hinton broth.)

(2) *Virological and mycological.* Acute serum for virological screening (at least Herpes simplex and Cytomegalovirus) and for detection of antifungal antibodies (at least *Candida* and *Aspergillus*). Technique used should be specified.

(3) *Biochemical.* Serum creatinine, blood urea, uric acid, electrolytes (Na^+, K^+, intracellular K^+, Cl^-, HCO_3^-), transaminases (SGOT, SGPT), alkaline phosphatase.

(4) *Hematological.* Hematocrit, hemoglobin, total WCC, differential WCC, platelet count.

(5) *Immunological.* Take, separate and store serum from 10 ml blood in deep freeze.

(6) *Radiological.* Chest X-Ray.

(b) *During treatment:* (1) *Bacteriological.* Repeat after 72 hr, including 1 × blood culture, then twice weekly.

(2) *Virological and mycological.* Once weekly.

(3) *Biochemical.* Twice weekly.

(4) *Haematological.* Twice weekly.

(5) *Immunological.* Once weekly.

(6) *Radiological.* Repeat chest X-ray at 72 hr if initial X-ray was negative and no other site of infection found.

(7) *Control of antimicrobial therapy.* Serum for back-titration against the offending pathogen; to be obtained 1 hr and 6 hrs after the administration of antibiotics on the second day of treatment.

(c) *After treatment:* (1) *Bacteriological.* Once (without blood cultures).

(2) *Virological and mycological.* Once.

(3) *Biochemical.* Once.

(4) *Hematological.* Once.

(5) *Immunological.* Once.

(6) *Radiological.* Once.

Notes. Other investigations should be done when and where indicated.

For patients receiving granulocyte transfusion 1 hr and 12 hr post transfusion white counts are taken; the HLA-type of both patients and donors should be determined.

For patients dying during treatment or during the follow-up period a complete autopsy report (including histologic findings) and results of cultures of heart, blood, spleen and lungs (and relevant lesions) should be attached to the final report form.

Temperature should be recorded 6-hourly during the period of treatment. Photocopies of the patient's temperature chart must be attached to the final report form*.

Bacteriological evaluation. Microorganisms isolated from the blood (bacteremic patients) should be sent to the reference laboratory (Institut Jules Bordet) with samples of serum (5 ml) obtained 1 hr and 6 hr after the injection of amikacin plus carbenicillin (and cefazolin). *In vitro* synergism and minimum inhibitory concentrations for the antibiotics given to the patient from whom the microorganism has been isolated are deter-

*Official report forms are provided to the participating Centers along with the randomization cards.

mined along with the antimicrobial activity of the serum against that organism.

Clinical evaluation should be performed by the principal investigator at the center 4 days after start of therapy and at the end of the therapy. The notes should be brief but describe accurately the status of the patient and the opinion of the attending physicians.

(C) *Granulocyte transfusion trial*

(a) *Definition of the poor prognosis group.* The criteria for inclusion in the poor prognosis group are listed on the randomization card. These criteria include serum creatinine level, level of neutropenia and thrombocytopenia, and height of fever. They must be confirmed by a bone marrow examination, showing a hypocellular bone marrow as defined earlier.

(b) *Administration of granulocytes.* When demanded by randomization, granulocytes are started within 24 hr after the onset of therapy with amikacin + carbenicillin (see below). They are administered daily for at least 4 consecutive days. The daily administered dose must exceed 10^{10} cells/m^2. The method of collection of granulocytes is unimportant, but *should be consistent* in the same patient. The control patients should not receive granulocytes during the first 96 hr of therapy with antibiotics unless their clinical condition continues to deteriorate following initiation of the protocol antibiotics. Addition of granulocytes during that time would mean failure of antibiotics alone.

(D) *Antimicrobial therapy trial*

(a) *Dosage. Amikacin.* 15 mg/kg daily in 2 divided doses; for the average adult give 500 mg/12 hr.

Carbenicillin. 500 mg/kg daily in 4 divided doses; for the average adult give 10 g/6 hr. Ticarcillin may be used instead of carbenicillin at a dose of 250 mg/kg daily; for the average adult give 5 g/6 hr.

Cefazolin. 50 mg/kg daily in 4 divided doses; for the average adult give 1 g/6 hr.

(b) *Mode of administration.* All the antibiotics are given intravenously. The i.v. injection is given in 100 ml of water and administered over a 15 min period. The antibiotics should never be mixed together. Each dose of amikacin should be administered immediately following the carbenicillin (and cefazolin) injection(s). Pediatric doses of the antibiotics should be given in a proportionately reduced volume of water.

(c) *Duration of therapy.* Antibiotics should not be changed during the first 5 days of therapy unless there is microbiological evidence of inadequate coverage *associated with* an inadequate clinical response. The response to trial antibiotics should then be considered as a failure. After 5 days of treatment, inappropriate antibiotics, on the basis of susceptibility tests, may be discontinued. If pathogens resistant to the antibiotics in the trial regimen are isolated and considered as significant pathogens, addition of new antibiotics is allowed, as indicated by susceptibility tests. Adding new antibiotics means that the response to the trial antibiotics is a failure.

If no significant pathogen has been isolated, the trial regimen should be continued for at least 5 days after temperature has returned to normal. If no clinical response has occurred, other agents may be added to the regimen if clinically indicated, but the trial regimen must be continued until 5 days of treatment have been completed.

If the patient has no infection (infection doubted), stop all antibiotics at 5 days and observe. Antibiotics should be stopped even if the patient is still febrile provided no other evidence of infection is present on thorough repeated history, physical examinations, chest X-rays and laboratory investigations.

(E) *Optional additional measures*

These measures (oral antibiotics, isolation, vaccine, etc), if used, must be applied equally and without preference to each treatment group in the same way and for the same length of time.

(F) *Definitions*: (1) Documentation:

1 = Microbiologically documented. Signs and symptoms of infection present (i.e. primary site of infection recognised) and positive bacteriological cultures obtained from the suspected site, blood cultures, or histological sections.*

2 = Clinically documented. Site of infection identified and progress consistent with infection. Negative cultures from primary site and blood.*

3 = Possible infection. Signs, symptoms and progress are consistent with infection. Negative cultures and no site found despite complete re-examinations, history, X-rays and cultures at least every 3 days.

4 = Infection doubted. Infection improbable on review of clinical signs and progress.

*e.g., a pulmonary infiltrate is a pneumonia if consistent with proper signs and symptoms of infection—*clinically documented*—but is *microbiologically documented* if (1) blood cultures are positive, (2) sputum culture is positive with a clear preponderance of one organism, the specimen is of good quality (i.e., not saliva), etc., or (3), positive results are obtained from transtracheal puncture, biopsy, etc. . . . Pneumonia remains *clinically documented* if blood cultures are negative or no blood cultures were taken and sputum sample was of poor quality or had mixed flora.

(2) Response to therapy: (clinical evaluation at 4 days and after discontinuation of protocol).

1 = Improved.† Lasting return of temperature, signs and symptoms to normal or to pre-infectious state.

2 = Temporary improvement. As for "Improved" but with relapse in 3–6 days despite continuing antibiotic therapy.

3 = Failure. Infection persists or patient dies or is treated with other antibiotics.

4 = Not evaluable. Patient improves or remains unchanged but response cannot be related specifically to antibiotics or granulocyte therapy. Also if fungal or viral infection, or infection doubted.

†*EXAMPLES*

Pneumonia. Improvement requires patient to become afebrile, have resolution of pulmonary signs and symptoms and to show resolution of pulmonary infiltrate, although X-ray clearing may lag behind clinical response.

Urinary tract infection. Improvement requires resolution of fever, signs and symptoms and at least one negative culture after stopping antimicrobial therapy.

Bacteremia. Improvement requires at least one negative blood culture after cessation of antibiotics.

A superinfection (e.g., *Aspergillus* pneumonia on top of primary *E. coli* pneumonia) may make evaluation impossible by the above criteria. In such a case consider the *E. coli* pneumonia improved if signs and symptoms were improving, pulmonary infiltrate was clearing, blood cultures were negative, etc. . . ., when new pneumonia supervened.

RESULTS AND ASSESSMENTS

Clinical reports should be completed and sent to the Statistical Center within 2 weeks of completion of treatment.

PUBLICATIONS AND LECTURES

Nothing shall be published and no lectures shall be given by any of the participating members of the Group about either the work or the results of the trial without consent of the Group. However, each participant is free to publish data from his own patients and wards.

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Encephalopathy Following Bone Marrow Transplantation

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Abstract—*Increasing numbers of bone marrow transplants are now being attempted for the treatment of acute leukemia. A case of encephalopathy following syngeneic marrow transplantation is described in which the main causative factor was thought to be the total body irradiation given as part of the conditioning regimen prior to transplantation. The possible cumulative risk of encephalopathy and myelopathy in patients previously given cranial or craniospinal irradiation and intrathecal methotrexate is discussed.*

INTRODUCTION

Several hundred bone marrow transplants for the treatment of acute leukaemia are currently being performed each year [1]. A number of these patients, especially those with acute lymphoblastic leukemia (ALL), have previously received cranial or craniospinal irradiation and/or intrathecal (IT) methotrexate (MTX). Total body irradiation (TBI) is frequently employed in an attempt to eradicate residual leukaemia prior to marrow transplantation. Whilst encephalopathy due to cumulative doses of MTX [2, 3], cranial irradiation [4], and a combination of IT cytotoxic chemotherapy, cranial irradiation and central nervous system (CNS) leukaemia [5] is now well recognized in children receiving conventional therapy for ALL, its occurrence due to the additive effect of such factors and TBI in patients undergoing bone marrow transplantation has not been previously described [6], although the potential risk has caused concern.

MATERIAL AND METHODS

Case report

A boy aged $3\frac{1}{2}$ yr had ALL diagnosed in September 1974. He was a monozygotic twin and his sibling was not affected. His initial blood count was: haemoglobin 100 gm/l., white count 160×10^9/l., with 80% lymphoblasts, 14% lymphocytes and 10% neutrophils. His platelet count was $45 \times 10/^9$l. Chemotherapy with prednisone, vincristine and 6-mercaptopurine produced a partial remission with return of the peripheral blood to normal, but 4% blast cells remained in the bone marrow. It was felt that these blast cells were leukaemic. At this time the cerebrospinal fluid (CSF) was normal, and prophylactic cranial irradiation (2000 rad in $2\frac{1}{2}$ weeks) together with 5 IT injections of MTX (10 mg/m^2) was given. This course of radiation was given from a 230 kV machine using fractions of 400 rad twice weekly for $2\frac{1}{2}$ weeks, at a dose rate of 78 rad/min. A 2 mm Cu. H.V.L. filter was used; the focus skin distance was 50 cm, and the separation between the two fields was 15 cm. The course was started on October 25, 1974 and finished on November 8, 1974. Apart from some post-irradiation somnolence lasting 2 weeks, and occurring 5 weeks after the end of prophylactic cranial irradiation, he remained well, but a bone marrow aspirate in December 1974 showed relapse. Further chemotherapy with prednisone, vincristine, 6-mercaptopurine, MTX, daunorubicin, cytosine arabinoside and asparaginase failed to produce a remission. Re-examination of the CSF at this time (December, 1974) showed blast cells to be present and he received a further four injections of IT MTX. Because he had an identical

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twin and was clearly doing badly it was decided to carry out a bone marrow transplantation. On February 10, 1975 after preparation with cyclophosphamide 60 mg/kg on two consecutive days followed three days later by TBI of 1000 rad in $3\frac{1}{2}$ hr from a ^{60}Co machine using a dose rate of 5 rad/min, he received bone marrow taken from his identical twin brother. He progressed satisfactorily, obtained a functioning graft, and bone marrow aspirate showed no evidence of leukaemia.

One month after bone marrow transplantation (March 1975), he gradually became drowsy and irritable, developed slurred speech and his gait was unsteady. Examination showed a right foot drop and increased tone in both legs but nothing else abnormal. The CSF, skull X-ray and brain scan were all normal, but the EEG showed grossly abnormal diffuse bilateral slow activity in all areas. Serum taken two months after the transplant contained no antibodies against measles or papovavirus; the titre against cytomegalovirus (CMV) was 1/32, against mycoplasma 1/32, and against influenza A 1/64. He became progressively more unresponsive, mute and, in addition to dementia, developed a spastic quadriplegia. This state persisted with little change for eight months. He also had occasional myoclonic jerks which coincided with bilateral spike and wave discharges on the EEG, apparently of subcortical origin.

Ten months after transplantation, with the bone marrow still in remission, he died with pneumonia, and at autopsy the brain showed macroscopic evidence of widespread leucoencephalopathy. This was confirmed by histological examination, which showed areas of demyelination and glial cell loss—changes similar to those described by Rubinstein [6]. Cultural and histological examination revealed no evidence of infection of the CNS by either bacterial, viral or protozoal agents.

DISCUSSION

There are several possible causes of the encephalopathy that he developed. IT MTX can produce subacute encephalopathy [2] manifested by somnolence, confusion, irritability, tremor, ataxia and fits. In this series by Kay *et al.* brain irradiation was not essential to produce the syndrome, which was associated with a partial improvement with folinic acid. In one autopsied case numerous small infarcts in the frontal, perietal and temporal lobes were present, together with necrosis of blood vessel walls. It is unlikely that IT MTX alone was the cause of encephalopathy in our patient, since, in Kay's series, all 7 patients had had massive amounts of oral, i.m. or i.v. MTX (944–4458 mg) prior to IT MTX. Our patient, apart from 60 mg IT MTX, received in addition a total of only 15 mg of oral MTX. Furthermore, encephalopathy in the reported cases developed during courses of IT injections. The neurotoxicity of IT MTX is associated with high CSF levels of MTX [7], and in the report by Bleyer *et al.* almost all cases of severe neurotoxicity occurred in patients with neuroleukaemia, suggesting that the latter may interfere with the egress of MTX from the CSF [8].

Cranial irradiation of 2400 rad by itself produces a transient encephalopathy, as in our own case, in a majority of children. This syndrome is characterized by somnolence, lethargy and anorexia [4]: recovery, however, is invariable. Combined IT cytotoxic therapy and irradiation in patients with neuroleukaemia or neurolymphoma can produce a subacute progressive encephalopathy [3, 5, 9] characterized by lethargy, irritability, dysphagia, dysphasia, spasticity and sometimes death.

A similar encephalopathy may occur as a consequence of measles during antileukaemic therapy, with or without a history of clinical measles, but with either a high antimeasles antibody titre or histological changes characteristic of disseminated measles infection [10]. This is an improbable cause in this case, although the role of the other viruses cannot be entirely excluded. The CMV titre is ambiguous but there was no other evidence of CMV infection.

The outstanding features that the majority of patients with such severe neurological complications share is (a) therapy with IT cytotoxic agents invariably including MTX, plus (b) whole brain irradiation, plus (c) malignant disease involving the CNS. Our own case was typical; not only did he receive IT MTX and have neuroleukaemia, but he underwent two courses of irradiation that included the brain—firstly, conventional prophylactic therapy (2000 rad in $2\frac{1}{2}$ weeks), and, 3 months later, 1000 rad in $3\frac{1}{2}$ hr. The biological equivalent of the large single dose is further complicated by the low dose rate at which it was delivered (5 rad/min) [11]. Reducing the dose rate to this level probably does reduce the degree of radiation damage in some normal tissues, particularly gastrointestinal mucosa [12]. Normal tissue sparing by reduction of radiation dose rate is more likely to occur in tissues which exhibit a marked capacity for

intracellular repair of sublethal radiation damage. Whether or not this might be the case in cerebral tissue is unknown. The timing in our case may have been important: radiation encephalopathy occurs several weeks to several months after irradiation, and it is possible that the large single dose may have been given during a critical period of the recovery of brain and vascular tissue from the first course of radiation. Age may also be important: our patient was $3\frac{1}{2}$ yr old, the ages of the children in Rubinstein's series [5] were $2–8\frac{1}{2}$. Younger children tended to have more severe symptoms in Freeman's series [4] of children given prophylactic cranial irradiation. The brain does not approach its adult weight until the age of 12: between the ages of 14 months and 12 yr there is an enormous increase in the weight of the brain (43%) and this is in part a reflection of myelin production [13]. The Seattle group have given 1000 rad TBI to 14 patients with acute leukaemia undergoing bone marrow transplantation, who have previously had cranial irradiation, and have had no cases of chemo-radiation encephalopathy [6]. Their youngest patient was 4 yr old.

The possible occurrence of this syndrome must be borne in mind when TBI of this nature is given to young marrow graft recipients who have had previous brain irradiation, IT cytotoxic chemotherapy and leukaemic involvement of the CNS.

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Interactions of Concanavalin A with Cell Surfaces of Normal and Tumor Rat Glial Cells Monitored by Agglutination and Cytochemical Detection*†

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Abstract—*Binding of concanavalin A to cell surface saccharides of rat glial cells, both normal and neoplastic, was investigated by two methods. Concanavalin A-mediated agglutination was demonstrated with tumor cells but not with normal newborn cells tested under the same conditions. Ultrastructural visualization of concanavalin A receptors by means of horseradish peroxidase revealed an essentially discontinuous type of staining on both tumor and normal cells alike, the distribution of the label being less evenly spread in the latter cells than in the former. Therefore, the observed difference in concanavalin A-induced agglutinability between normal and tumor glial cells did not correlate with whatever difference was detected by the cytochemical reaction in the distribution of concanavalin A binding sites between the two populations of rat glial cells. In conclusion, it seems reasonable to argue that the non-random distribution of cell-bound concanavalin A bears little or nothing upon the positive lectin-induced agglutination behaviour of the tumor cells, being apparently insufficient for or coincidentally related to it.*

INTRODUCTION

CONCANAVALIN A (Con A), a lectin with certain saccharide-binding specificities, has been widely applied as a molecular probe for the study of the cell surface (for literature see [1]). We used Con A for investigating the plasmalemma of normal and tumor glial cells of rat, in order to test whether any differences could be detected between these two cell populations and whether such differences could be correlated with the normal and neoplastic state. The results of our studies are reported in this communication; their significance is briefly discussed.

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MATERIAL AND METHODS

Tumor cell cultures

Seven gliomas induced in rats by systemic application of methylnitrosourea [2] were dissected aseptically, rinsed in calcium- and magnesium-free phosphate buffered saline (PBS) solution, trimmed down to appropriately small fragments, explanted into Falcon plastic tissue culture vessels and grown routinely at 37°C in M-199 medium (Seromed, Munich, FRG) supplemented with 10% fetal bovine serum (Seromed, Munich, FRG). Primary cultures were subcultured many times. Passages 5, 7 and 9 of each cell line were used for agglutination and cytochemistry studies.

Normal cell cultures

The intracranial structures of newborn rats were exposed under aseptic conditions. Small

tissue fragments were excised from the cerebral hemispheres and cerebellum and rinsed with PBS. The fragments were seeded in plastic tissue culture vessels and grown routinely at 37°C in M-199 medium supplemented with10% fetal bovine serum. Ten primary cultures were established. They were subcultured several times. The morphology of the subcultures followed by light microscopy revealed that most of the cells had an epithelial aspect. Passages 5, 7 and 9 of each cell line were used for agglutination and cytochemical control studies

Agglutination test

Normal and tumor cells were detached after draining the medium and washing the cell layer with PBS. Following the third and last rinse the cells were flushed loose to a single-cell suspension with PBS pipetted gently against the bottom of the tissue culture vessel supporting the cell sheet. Cell viability by trypan exclusion test was more than 90%. Chemical methods of detachment employing proteolytic or chelating agents were discarded in view of the untoward effects on the cell membrane composition [3] and function [4] observed after using these substances. Equal volumes of cells suspended in PBS at a density of 10^6 cells/ml were mixed with equal volumes of PBS solutions of Con A (Serva, Heidelberg, FRG) of decreasing concentrations. These had been prepared prior to usage by diluting serially a 0·1% stock solution of Con A with PBS at dilution ratios ranging from 1:2 to 1:2048. The cell-Con A mixtures were finally incubated at 37°C for 30 min. The results were checked with the naked eye and then by placing a drop of the incubated mixture on a slide and examining it under the 16 mm objective of the microscope. Agglutination was scored using a semiquantitative scale ranging from ± to + + + +, the latter value indicating that all cells (100%) in the tested mixtures had been trapped into clumps.

Some cultures of normal and tumor cells were harvested as described above, exposed to 0·25% trypsin (Serva, Heidelberg, FRG) for 20 min and then incubated with Con A as the remaining cultures. The specificity of the phenomenon was checked by incubating additional cell cultures with a Con A solution containing 0·2 M of methyl-alpha-D-manopyranoside (MAM) (Sigma, Munich, FRG).

Cytochemical reaction

Con A staining of the cell surface was carried out on living cells *in situ* according to a slightly modified version of the original method described by Bernhard and Avrameas [5]. The medium was decanted and the cells, both normal and neoplastic, were first washed with PBS and then incubated with a 50 μg/ml PBS solution of Con A for 15 min at room temperature. Whereupon, the cells were rinsed again with PBS and incubated with a 50 μg/ml PBS solution of horseradish peroxidase (HRP) (Sigma, Munich, FRG) for 15 min at room temperature. Subsequently, the cells were washed with PBS and fixed for 15 min with a 2·5% PBS solution of glutaraldehyde. After renewed washing in PBS the cells were incubated for 15 min with a 500 μg/ml PBS solution of diaminobenzidine (Sigma, Munich, FRG) to which a few drops of a 0·1% solution of H_2O_2 had been added just prior to usage. Following rinsing with PBS the cells were postfixed for 1 hr with a 1% OsO4 solution, dehydrated in graded ethanol solutions and embedded in Durcupan. The plastic flasks were broken, the Durcupan layer was detached and appropriate areas of it were chosen under the microscope for further processing. Ultrathin sections were prepared with diamond knives, double stained with uranyl acetate and lead citrate and viewed with a Zeiss EM 9A electron microscope. The specificity of the reaction was controlled by adding 0·2 M of MAM to the Con A and HRP solutions of some cultures in each experiment. In addition, the effect of prefixation on Con A binding sites was tested by treating some cultures with 2·5% glutaraldehyde for 15 min prior to addition of Con A. The effect of trypsinization on the distribution of cell-bound Con A receptors demonstrable cytochemically was not tested

RESULTS

Agglutination test

All lines, regardless of their derivation from normal or neoplastic cells, were maximally agglutinated at the higher concentrations of Con A. Therefore, the data reported hereafter refer exclusively to results obtained under constant conditions of cell density, temperature, incubation time and Con A concentration, the latter corresponding namely to the 1:32 dilution.

Under the above-mentioned standard conditions, no agglutination was observed with untreated normal cells. Trypsinized normal cells showed a degree of agglutination corresponding to a 75% reduction or more in the number of single cells. A comparable + + +

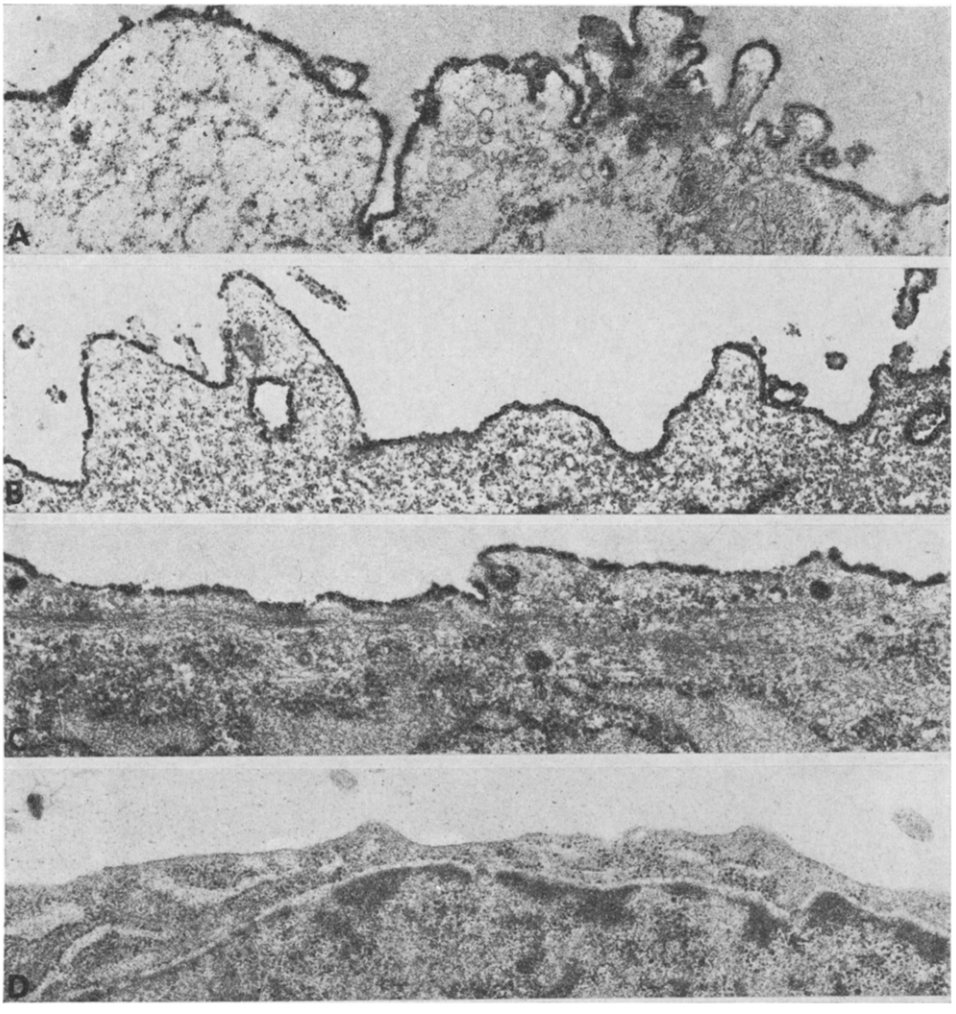

Fig. 1. *Rat glial cells stained with Con A-HRP either* in situ *and after glutaraldehyde fixation* (*A*) *or* in situ *and intra vitam before any such fixation* (*B, C, D*).

(*A*) *Segment of a tumor cell fixed* in situ *prior to incubation with Con A and HRP: the electron dense material forms almost a continuous ribbon decorating the cell surface* (× 21000).

(*B*) *Segment of a tumor cell stained intra vitam: a few isolated breaks interrupt the continuity of the surface label* (× 18000).

(*C*) *Segment of a normal cell stained intra vitam: solutions of continuity along the superficial band of reaction product are numerous and of varied extent* (× 24000).

(*D*) *Segment of a tumor cell stained intra vitam with Con A and HRP solutions to which MAM had been added: no surface staining* (× 21000).

agglutination was obtained with untreated tumor cells and trypsinized tumor cells alike. No agglutination was detected around the multinucleated giant cells occurring isolatedly in the cultures of experimental rat gliomas. Tumor cells, either untreated or trypsinized, and trypsinized normal cells did not agglutinate whenever MAM had been added to the lectin solution. No essential differences were detected between the different lines of normal cells and the different passages of the same cell line. Likewise, no essential differences were observed between the different lines of tumor cells and the different passages of the same cell line.

Cytochemical reaction

An almost continuous type of staining with few, small breaks thinly scattered along the ribbon of electron dense material was seen in the prefixed normal and tumor cells (Fig. 1A). Making allowances for a considerable degree of intercellular variation, untreated normal and tumor cells alike had less than a continuous distribution of the reaction product on their surfaces. Most of these cells showed lengths of continuous label following areas with a dotted or dot-and-dash type of label, and alternating with either bare spots or denuded patches of varied extent. In general, irregularities of the stained surface and discontinuities of all sizes were less numerous and extended over shorter stretches in tumor (Fig. 1B) than in normal (Fig. 1C) cells. Normal and neoplastic cells alike showed no surface staining (Fig. 1D) whenever MAM had been added to the Con A and HRP solutions. The results were essentially the same in the different cell lines and in the different passages of the same line.

DISCUSSION

We will discuss the results of the agglutination assay and cytochemical reaction in that order; at the end, we will briefly consider the two sets of findings taken together.

The results of our agglutination tests are in line with those of a large series of observations (for review see [1]). They are at variance, however, with those reported by Glimelius *et al.* [6] who also had been experimenting with cultures of normal and tumor glial cells. These authors found essentially no difference between normal and tumor cells, all cells being invariably agglutinated in presence of Con A at weaker concentrations and shorter incubation times than those used in our experiments. The reasons for the reported discrepancy between our results and those of the Scandinavian workers are not clear. It is possible that the difference pertaining to the animal species from which the tested cells had originated (rodent vs human) and that concerning the handling of the cells prior to incubation (PBS vs EDTA) might account entirely for the divergent results.

The essentially irregular distribution of the reaction product observed on the plasmalemma of our normal and tumor cells conforms to that reported by earlier observers in other pairs of normal cells and their modified counterparts [7–9]. It is at variance, however, with that reported by the same [7] or by other workers [10–12] in still other pairs of cells. In this connection it should be mentioned, however, that of all ultrastructural studies of this type none have dealt with glial cells of any kind, so that a meaningful comparison between the results yielded by rat glial cells in our hands and the results yielded by glial cells of either rat or another species in the hands of other authors is still awaiting. In the meanwhile, it is evident that an essentially discontinuous type of Con A labeling is neither characteristic of glioma cells nor inconsistent with normal newborn glial cells; whether also normal adult glial cells have a discontinuous type of labeling remains to be proven. For the time being it appears that rat glial cells, both normal and neoplastic, have an essentially similar type of labeling, and that staining is likewise irregular in both normal and tumor cells, being only less evenly spread in the former cells than in the latter. It seems further that the pattern we observed in our glial cell pair cannot be expected to have general validity nor to occur regularly, although it might be seen in other cell populations probed for binding sites with Con A or with another lectin.

Finally, if the distribution of the Con A receptors brought to light by the cytochemical method is largely similar in normal and tumor rat glial cells alike, then it shows no correlation with the negative and positive agglutination responses of these same normal and tumor glial cells, respectively, when incubated with a Con A solution of the appropriate concentration. Some authors [13, 14] investigating the Con A binding sites of other pairs of cells and getting essentially the same results as we did, concluded that unevenly distributed cell-bound Con A has little or no effect upon the lectin-induced agglutinability of the tested cells: a conclusion with which we would tend to agree, if we had to judge from the results of the work presented in this communication.

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Announcement

SYMPOSIUM ON: CLINICAL APPLICATION OF CARCINO-EMBRYONIC ANTIGEN AND OTHER ANTIGENIC MARKER ASSAYS

7–8–9 *October* 1977

Sponsored by the European Organisation for Research on Treatment of Cancer. Organized by the Centre Antoine Lacassagne, Nice.

Direct inquiries and proposals of papers to:

Mrs. F. Fein, secretariat Symposium "Tumour Antigens", 36 Voie Romaine, 06054, Nice Cedex, France. Tel: (93) 80.54.23 Ext: 248. Telex: CAL NICE 470 344 F.

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Perspectives in Cancer Research

Present and Future Clinical Relevance of Tumour Markers*

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INTRODUCTION

THERE has been growing awareness, during the past 10 years, of the ability of neoplastic tissue to synthesize and release into the circulating blood, substances that are either lacking or are produced in extremely limited amounts by the original parent cells. The appearance of these substances, referred to as tumour markers or cancer-related antigens (due to their protein and polypeptide nature), is most often interpreted as being the result of derepression of silent genes during the processes of dedifferentiation and disorganization which accompany the malignant transformation.

These tumour markers have attracted considerable attention from biologists, biochemists, immunologists and clinicians for several reasons. In the first place, these antigens of cancerous origin may be expected to play an important role in the natural defense of the organism against the development of cancer. In fact, according to Currie [1] "It is clear that most, if not all, animal and human tumours possess cell membrane antigens which are immunogenic in the host".

They may be directly implicated in the rejection or the tolerance of the cancer by the host. Besides, these tumour antigens and the immunological reaction they elicit in the host may constitute one of the factors controlling the kinetics of multiplication of the cancer cells and thereby the growth of the tumour. Furthermore, the immunogenic character of these antigens and the localization on membranes of a good number of them can constitute the basis for specific active immunotherapy—indeed, they already do. Finally, the assay of the antigens in biological media such as blood, urine and the cerebrospinal fluid [2] serves as a useful method not only in the diagnosis but also in the determination of the extension of the cancer and in its therapeutic surveillance as well.

Our objective in this editorial is to: (1) list the principal cancer-related antigens and (2) define such properties and limitations of these markers as would be relevant to the clinician interested in their use for diagnostic purposes as well as for assessing tumour extension and the effectiveness of therapy.

We will conclude with an analysis of the directions of future research concerned with tumour markers.

1. CLASSIFICATION AND NATURE OF TUMOUR MARKERS

If one were to generalize on the properties of the various substances that have so far been used as tumour markers, one could state the following:

(a) Most tumour markers known to date are protein or polypeptide in nature since immunological methods have enabled their identification, purification and isolation. Nevertheless, it is equally possible that numerous other substances that are not protein in nature and that are more or less specific for cancer cells are also secreted. The polyamines constitute such an

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example—while they are not specific to the cancerous state, they are synthesized in large excess and released into the blood by growing tumours.

(b) They may belong to several parts of cancerous cells: sometimes to the nuclear, but most frequently to the cytoplasmic and membrane structures.

Their association with membranes confers a double interest: the antigen appears more readily in the blood and induces an immunological reaction which could be of great pathophysiological significance. Whether of cytoplasmic or nuclear origin release into the circulation and eventual induction of immunological reaction are equally feasible. Some substances which are sometimes used as tumour markers, belong to the normal tissue invaded by the neoplasms or by the metastases or are consequent upon the inflammatory reaction accompanying the tumour. Specificity of such markers is always very poor and only dependent on the quantitation of their assay.

(c) They can be biologically active and induce clinical symptoms as in ectopic hormone syndromes. However, the majority of tumour markers do not possess any biological activity; this does not exclude the possibility of a role in the tumour-host interaction, nor in the non-specific clinical manifestations such as cachexia nor in the modifications of the immunological defence, etc.

(d) On the basis of physico-chemical, immunochemical and biochemical analogies which exist between these tumour markers and substance normally found at particular times of life, one can offer a classification based on different categories:

Table 1. *Foetal and placental antigens*

Antigen	Foetal tissue of origin	Identified in blood of cancerous patients	Principal associated tumour
I. *Foetal antigens*			
Carcinoembryonic antigen (CEA)	G.I. tract	+	About 50% of all tumours
α Foetoprotein (αFP)	Serum, liver G.I. tract	+	Hepatocellular, teratomas
γ Foetoprotein (γFP)	Serum, G.I. tract spleen, thymus	+	11% various tumours
α_2H Ferroprotein (α_2HFP)	Liver	+	Leukaemias, Hodgkin's
Carcino-foetal glial antigen	Brain	After immunization	Gliomas
Foetal sulphoglycoprotein	G.I. tract	−	Gastric juice in stomach carcinoma
Melanoma-associated antigen	Melanoma	?	Melanoma and other various tumour
β Oncofoetal antigen	Fetal organs	+	All types of carcinomas, some cases of lymphomas
Leukaemias-associated antigen	Serum and various tissues	+	Leukaemias, Hodgkin' disease
II. *Placental antigens*			
Human chorionic gonadotrophin (HCG)	Trophoblast	+	Choriocarcinoma, teratomas, pancreatic cancer, 14% of various tumours
β Subunit of HCG (βHCG)	Trophoblast	+	16% of lung cancer, pancreas tumour
α Subunit of HCG (αHCG)	Trophoblast	+	Choriocarcinoma, teratomas 6% of digestive cancers 8% of lung cancers
Human placental lactogen (HPL)	Trophoblast	+	Teratomas, choriocarcinomas, various tumours
Placental alkaline phosphatase	Placenta	+	Various tumours Incidence: 1-12% in different series
Human chorionic thyrotrophin (HCT)	Trophoblast		Choriocarcinoma
Pyruvate kinase	Placenta	+	To be determined

1. *Foetal or embryonic antigens*

These substances are present in the tissues and serum of the foetus and either disappear or are drastically reduced in amount at the end of gestation or in the first weeks of extra-uterine life. Several foetal antigens are recognized (Table 1) but the two foetal antigens best characterized to date are carcinoembryonic antigen (CEA) and α foeto protein (AFP). CEA is a glycoprotein discovered by Gold and Freedman [3] in 1965 in the gastrointestinal tissues of the human foetus up to 6 months gestation and in adenocarcinomas derived from the gastrointestinal epithelium. The studies of Krupey *et al.* [4], Gold *et al.* [5] and von Kleist and Burtin [6], showed that it is a water-soluble membrane glycoprotein with a sedimentation coefficient of 7–8 S, electrophoretic mobility characteristic of β globulin and molecular weight of about 200,000 dalton. As shown by electronmicroscopy [7] CEA is present in the glycocalyx of the tumour cell. CEA displays a rich degree of polymorphism.

Human α foeto protein is made up of a single polypeptide chain with a molecular weight of about 70,000. It contains 4% carbohydrate and 2 mole of sialic acid per mole of protein [8]. It acts as a serum protein in the foetus since, synthesized by the foetal liver and by the yolk sac, it appears in the circulation from the 4th week of gestation. The serum concentration of AFP reaches its maximum levels, greater than 1 mg/ml, between the 12th and the 14th weeks of intra-uterine life. The total quantity of AFP in the foetus however increases up to 32 weeks and then falls [9]. Tatarinov [10] found AFP in the serum of a patient with a primary hepatoma and that finding has been confirmed subsequently on numerous occasions. The physico-chemical and immunochemical properties and the amino acid composition are identical for α foeto protein whether it be extracted from human foetal liver or from hepatoma.

Other foetal antigens have been described and are shown in Table 1. To this list should be added β-oncofoetal antigen and the melanoma cell membrane antigens. While these new antigens have been identified, we do not as yet have detailed information as to their frequency and specificity in relation to neoplasia and their presence in the circulating blood of cancer patients.

Beta oncofoetal antigen (BOFA) is a new antigen associated with several types of human cancer [11]. It was found to be different from AFP, CEA, ferritin and other previously described oncofoetal antigens. This new antigen seems to be present in many different types of human cancer at concentrations significantly higher than in normal adult tissues. A variety of new antigens expressed on the surface of melanoma cells have been described [12]. Some are tumour antigens and may be defined as re-expressed foetal antigens [13] whereas no clear conclusion can be drawn from the existing literature on the specific melanoma antigens as to whether they are common cross reacting antigens [14] or individually specific antigens [15].

2. *Placental antigens*

A second category of substances synthesized by the cancer cells are placental antigens which are normally present only in the serum of pregnant women. Amongst these are hormones such as human chorionic gonadotrophin (HCG) and its α and β subunits as well as human placental lactogen (HPL), all of which possess very specific antigenic determinants [16].

Enzymes constitute a group of non-hormonal placental antigens. Placental alkaline phosphatase has been investigated in depth but placental enzymes such as pyruvate-kinase appear to be of great potential interest in clinical and biological studies of cancers. In fact, immunological similarity exists between pyruvate-kinase from placental and from tumours of lung and stomach whereas no such similarity could be detected between pyruvate-kinase from placenta and several other normal human tissues.

Fottrell and Spellman [17] have demonstrated by immunological methods the appearance of placental pyruvate-kinase in the circulation of patients with tumours.

3. *Protein and polypeptide hormones*

Protein and polypeptide hormones constitute a third group of antigens produced by tumours. Numerous studies clearly demonstrate that various tumours are capable of secreting virtually all of the polypeptide hormones: ACTH, parathormone, calcitonin, ADH, etc. . . .

In principle, these ectopic endocrine secretions are of little interest in the diagnosis of tumours or the monitoring of tumour therapy because they are present under all physiological circumstances. They are not specific for the presence of tumour but there may be quantitative differences and it appears that the physico-chemical structures of ectopic hormones are different from those of hormones normally secreted by the relevant gland. Thus, a larger, more stable and more acidic form of ACTH which eludes before monomeric ACTH on gel

chromatography occurs consistently in tumour associated with the ectopic ACTH syndrome. In addition, this "big ACTH" is relatively resistant to suppression by steroid. Further characterization of the big ACTH molecule and the development of specific plasma assays would be of considerable clinical interest since it appears to be relatively abundant in patients with lung cancer [18]. Similar data were obtained with neurophysin [19]. The precancerous state might be capable of definition on the basis of the specific physico-chemical configurations characterizing such ectopic hormones. Certain antigenic groups could also be characterized by the ectopic hormone either following its unmasking or by an enhancement of its immunoreactivity in the new physico-chemical form. This may well be the case for calcitonin. Thus, with certain anti-sera and not others, it is possible to state that a majority of unselected breast carcinomas contained calcitonin as did some lung carcinomas [20, 21].

4. *Exocrine products*

A group of substances that have lately acquired relevance consists of exocrine secretion products. An example is casein, the phosphoprotein constituent of milk. This secretion of the breast is normally encountered only in pregnancy and lactation [22–24].

Lactoferrin and lactalbumin are also potential tumour markers, although recent investigations in clinical practice are unfortunately disappointing [25, 26].

5. *Normal cell constituents*

Different substances such as enzymes and polyamines are used as tumour markers. They are not specific in themselves but may acquire more significance because of their quantity. Tormey *et al.* [27] used N_2, N_2 dimethylguanosine associated with CEA and HCG in their study of breast cancer. In contrast putressin, spermidin, spermine, pseudouridine and I-methylinosine do not appear to be useful in the assessment of metastatic breast cancer or its treatment.

Gamma-glutamyl-transpeptidase in combination with CEA has been used by Cooper *et al.* [28] as a routine method of identifying hepatic metastases.

Other non-specific substances which have been proposed at some time or another as suitable markers to follow the evolution of cancer or suspected metastases include particularly haptoglobin, antitrypsin and acid glycoprotein [29].

2. CLINICAL RELEVANCE OF RADIOIMMUNOASSAY OF CANCER RELATED ANTIGENS

In order to draw conclusions in this field, five questions about the clinical value of the radioimmunoassays of tumour markers will be posed and answered.

1. *Are the cancer-related antigens specific for a particular type of tumour, or are they common to all neoplastic processes?*

Certain of these antigens are specific for a single organ and even for a single histological type of tumour. This is the case for α foetoprotein which is found almost exclusively in hepatoma and teratoma. Likewise, HCG and its α and β subunits are found in the serum of all the trophoblastic tumours and to a lesser degree embryonal tumours.

In contrast CEA has no specificity for any single type of tumour. It is found with approximately equal frequences in tumours developing from different sites: stomach, intestines, lung, breast, urinary tract, gonads, etc.

The frequency of kapa casein is greater in serum from patients with breast cancer particularly in the presence of metastases. This exocrine product appears to be a form of ectopic secretion of casein, as we have also found this substance in the serum of patients with cancers arising in the gastrointestinal tract and lungs.

2. *Is the radioimmunoassay of cancer-related antigens of value in early screening for cancer?*

At the present time the role of radioimmunoassays in the early diagnosis of cancer is impossible to assess. No epidemiological study has in fact been published.

From the biological point-of-view it is still not known at what stage of dedifferentiation of a tumour the antigens are secreted and how many cancer cells must be present before these antigens become detectable in the blood. From the practical point-of-view it must be established whether these substances occur in the blood earlier than clinically, radiologically and physically detectable morphological changes. The localization of the particular organ affected is obviously an important factor. In fact, if clinical investigations are difficult as in pancreatic carcinoma, biochemical diagnosis would be of greater importance than when the tumour commences in a tissue easily accessible to clinical investigation. It must be stressed that high risk factors for cancer are sometimes associated with cancer-related antigens, e.g. sera from

heavy smokers often contain CEA and big ACTH [30].

3. *Is the radioimmunoassay of these cancer-related antigens useful in making the diagnosis of neoplastic disorder?*

The usefulness of measuring these antigens in establishing the diagnosis of a neoplastic disorder merits discussion. If the quantitative nature of measurement is taken into consideration and if several markers are simultaneously assayed, we consider measurement of cancer-related antigens to be a useful diagnostic procedure, which, however, is neither absolute nor specific.

In a recent study [31] five tumour markers were measured simultaneously in serum by radioimmunoassay: CEA, AFP, HCG, β subunit of HCG and K-casein. Defining abnormal levels as those which were higher than maximun values detected in a population of 935 normal subjects, we confirmed that the incidence of positive findings for one of these antigens is 14.7% in non neoplastic disorders which limits the absolute specificity of these assays in establishing the diagnosis of cancer. It must, however, be recalled that the positive findings of these cancer associated antigens is particularly marked in liver disease and in acute pulmonary disorders [32–36], which can more readily be diagnosed by clinical examination and biochemical and radiological investigations. Thus, in the presence of pathological levels of these tumour markers, one must first of all exclude liver and acute pulmonary diseases of non-neoplastic origin before ascribing diagnostic significance to the results of the assays. False positivity may be partially controlled in this way.

Furthermore, the assay of these antigens is not always positive in cases of cancer at the beginning of the disease. Thus, the incidence of positivity of any one of these five cancer associated antigens was 72% in patients at the onset of the disorder, when clinical symptoms led the patients to consult for the first time, and in the absence of metastases. In fact, if CEA was considered alone its incidence was 53% in the general population of patients with cancer at the beginning of the disease. As shown in Fig. 1, the higher incidence is due to the additional estimation of casein and also of HCG and β HCG. Thus, the incidence of false negativity is decreased by the simultaneous measurement of several antigens.

It should be noted that AFP is only rarely found except in cases of liver disease, more particularly hepatoma. In these cases, it is always found together with CEA and/or β HCG. Thus, AFP would not appear to be an essential tumour marker in the screening of patients suspected to be suffering from cancer.

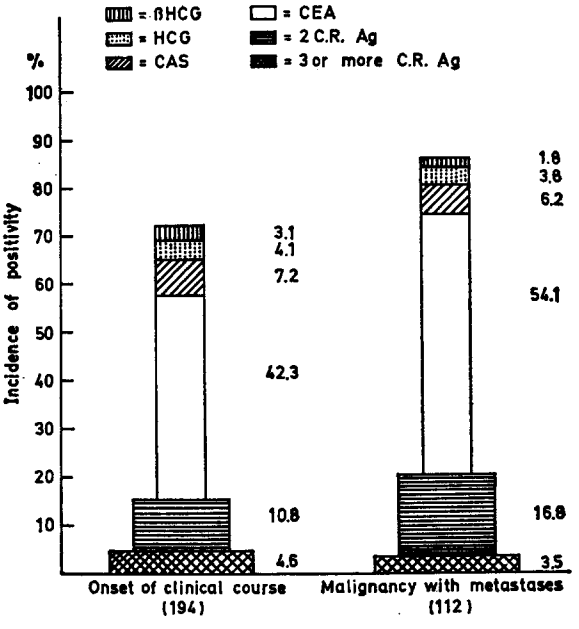

Fig. 1. Incidence of positivity of each cancer-related antigen in a sample of patients with malignancies at the onset of the clinical course, and with metastases. Horizontal bars indicate the incidence of positivity of 2 antigens and cross-hatched line the incidence of simultaneous positivity of 3 or more cancer-related antigens. Number represents the exact incidence.

As the detection of HCG and its two subunits is common to all trophoblastic tumours, the measurement of these substances is an important feature in the diagnosis of these tumours and may provide a means for early detection by screening. The ratio of α/β subunits is always lower than one as in early pregnancy but the quantity of HCG, β subunit is greatly increased in trophoblastic tumours [24]. The incidence of abnormal levels of HCG and its subunits is also high in embryonal tumours but only the levels of α subunit are invariably elevated.

In breast cancer, CEA and K-casein are most often increased and these associations may be of use in conjunction with other diagnostic investigations such as clinical examination, thermography and mammography.

Calcitonin may be of potential interest in breast cancer as it was shown with some selected antisera that the majority of unselected breast carcinomas contained this hormone [20]. Finally the combination of CEA and gamma-glutamy-transpeptidase (CGT) has been used as routine method for recognizing the presence

of hepatic metastases and for following their response to treatment by chemotherapy [28, 29].

4. *Does the concentration of cancer-related antigens provide information about extension of the neoplastic process?*

Here the reply is in the affirmative. Thus, when any cancer is complicated by metastasis the incidence of pathological levels and the absolute levels of some antigens such as CEA and kapa casein are higher than in those without metastasis. Furthermore, Laurence *et al.* [37] showed that the larger the primary tumour the higher is the level of CEA. The same relationship between the size of hepatoma and AFP levels was established by several authors [38]. Bagshawe [39] showed that the levels of production of HCG during the active phase of proliferation of trophoblastic tumour is characterised by exponential growth leading to doubling of the levels at intervals of 36 hr to 14 days depending on the individual patient and the nature of the tumour.

As an example, the levels of casein and CEA are higher in breast tumours complicated by metastasis or local recurrence than in such tumours when first diagnosed in the absence of metastasis (Figs. 2 and 3). Moreover, Chu and Nemoto [40] have established that the incidence of positivity and levels of tumour markers also varied according to the site of breast carcinoma metastases.

5. *Is the assay of cancer-related antigens useful in following the evolution of the tumour and the efficacy of therapy?*

The determination of the levels of these tumour markers provides a good criterion of the evolution of the neoplastic disorder and for following treatment. This was first demonstrated in gastrointestinal malignancy in which CEA is a useful index by which the completeness of surgical removal can be assessed: in cases of total ablation, CEA levels fall and remain undetectable. In cases of local recurrence or metastasis, CEA levels may fall to a greater or lesser degree but do not disappear. Ultimately, they increase sometimes several months before the metastases become clinically and radiologically evident [37].

Further, the usefulness of the measurements of certain antigens such as CEA, AFP and HCG in monitoring treatment has already been demonstrated by various authors [2, 33, 41–43]. The persistence of antigens in pathological concentrations appears to be correlated with the likelihood of recurrence. In our recent study on Breast Cancer [43] using 5 cancer-related antigens when the tumour had been removed at Stage N_0 the incidence of positivity of at least one antigen was only 10% whilst it reached 54% when the tumour had been removed but invasion of the regional lymph gland had already occurred (N^+). Further follow-up of the involved patients will be necessary to assess the values of the positivity as a precocious index of recurrence and/or metastases (Figs. 2 and 3). The estimation of CEA, HCG and N_2, N_2 dimethylguanosine in postoperative N^+ patients revealed one or more abnormalities in 67% [27]. Chu and Nemoto [40] also demonstrated in breast carcinoma that CEA levels after mastectomy were normal or transiently increased in 15 patients with no apparent metastases whereas increased values were detected in 4 out of 6 patients developing metastases during the follow-up.

In the group of patients treated by chemotherapy and radiotherapy the incidence of positivity of at least one cancer antigen is raised due to either local or general extension of the neoplastic process (which has led to the use of this therapy) or to the persistence of cancer cells which have escaped these varieties of treatment. It must be recalled that CEA levels fluctuate during chemotherapy, often in the same direction as the clinical course [41–44].

3. THE DIRECTION OF FUTURE RESEARCH

The study of tumour markers is still in its infancy. Only a few substances are known and even fewer are sufficiently pure to be suitable for radioimmunoassays. It is not unreasonable to postulate that there are other tumour markers, which are not necessarily proteins, which need to be identified, isolated and purified.

The limiting factor is without doubt the lack of specificity of known tumour markers. This lack of absolute specifity has several causes.

Firstly, methodology: in practice radioimmunological techniques are delicate and one must pay rigorous attention to fulfil the conditions necessary for their realisation, in particular to the purity of the tracer and its iodination, immunological specifity and the influence of non-specific proteins, etc. These technical qualities have not always been taken into consideration in certain studies and may account for anomalous results which are probably due to artefacts.

Secondly, biological factors: it is known that in practice repression of a gene is never complete. Thus, it is common to find traces of

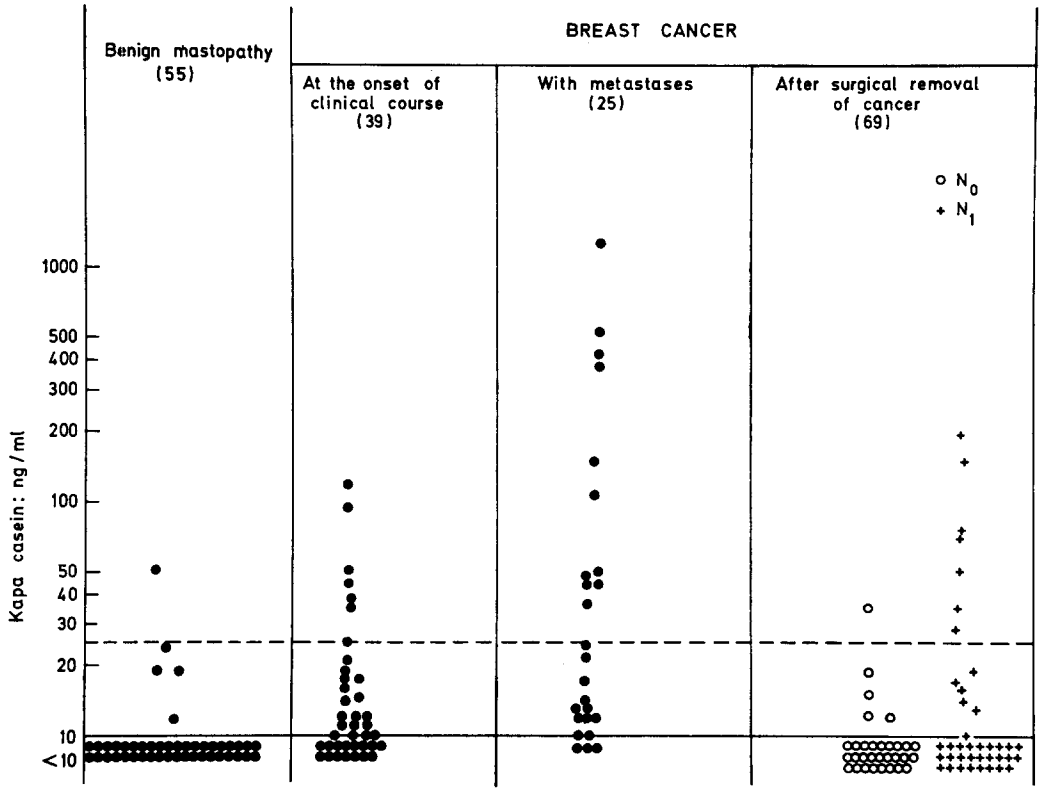

Fig. 2. Kapa casein levels expressed in ng/ml *in breast disease: benign mastopathy and breast cancer at different stages of evolution. In brackets, the number of patients.* N_0 *represents the casein rate when the tumour has been removed at stage* N_0*, i.e. without lymph node involvement.* N^+ *when, at the operation, regional lymph nodes were found to be invaded by cancer cells. The broken line represents the upper limit of normal.* 10 ng/ml *is the limit of sensitivity.*

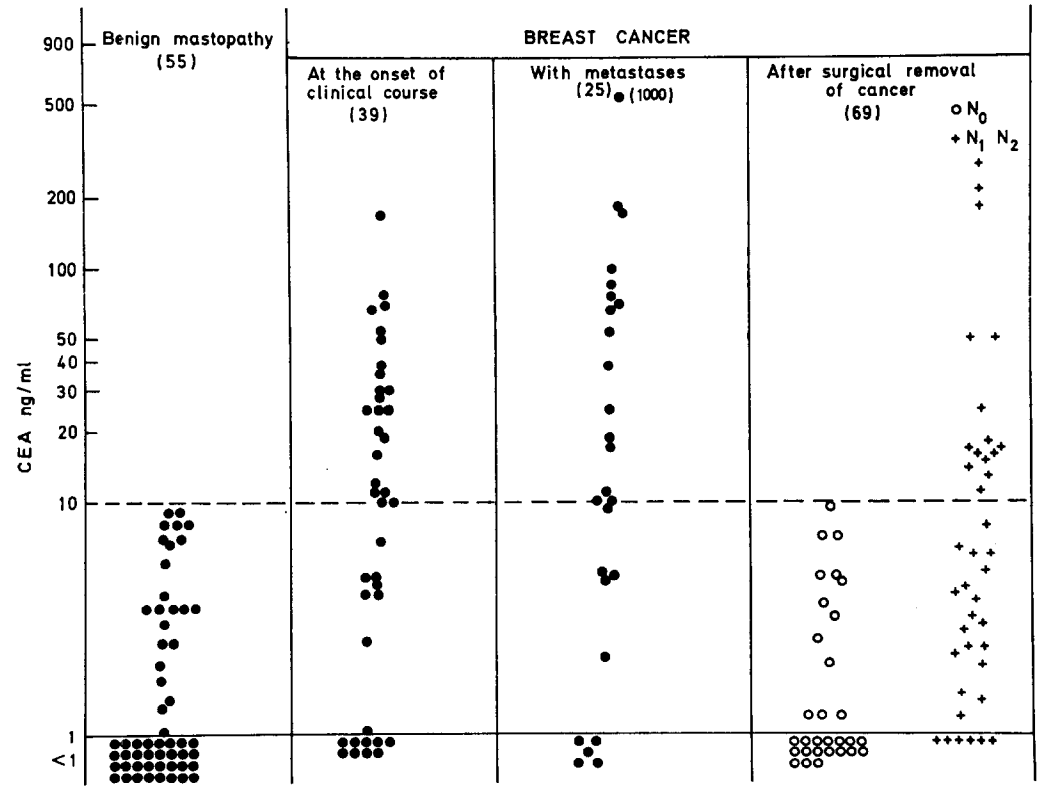

Fig. 3. CEA levels expressed in ng/ml *in breast disease: benign mastopathy and breast cancer at different stages of evolution. In brackets, the number of patients.* N_0 *represents the CEA rate when the tumour has been removed, at stage* N_0*, i.e. without lymph node involvement.* N^+ *when, at the operation, regional lymph nodes were found to be invaded by cancerous cells. The broken line represents the upper limit of normal,* 1 ng/ml *is the limit of sensitivity.*

embryonic antigens in several tissues [45] and sometimes in blood.

The specificity of the assays of cancer antigens could be improved in different ways. It is possible to select specific antigenic groups produced by the cancer cells, and to prepare corresponding antibodies. There appear to lie within the CEA molecule some common non specific antigenic groups belonging either to a glycoprotein to be found in the serum of normal subjects the NCA (non specific cross reacting antigens) isolated by von Kleist and Burtin [46], or to a glycoprotein secreted by the intestinal mucosa, found in meconium and also in the faeces of adults [47]. CEA produced by the cancer contains highly specific antigenic groups which should provide the basis for a specific radioimmunoassay of this embryonic antigen.

The recognition of hormonal exocrine forms relatively specific for neoplasia could lead to the development of specific plasma assays of exocrine and endocrine substance which would become much more interesting tumour markers than those available at present.

Another direction of research lies in the investigations of factors controlling the secretion of hormonal and exocrine products. Along these lines the use of dynamic tests provoking the liberation of tumour markers provides more useful information than that obtained by estimating only their basal levels. Furthermore it should be possible to study *in vivo* the hormone dependence of certain cancers by observing the rate of production of tumour markers under the modifying influence of hormones which exert an action on the normal tissue from which the tumour derives.

Finally, whilst the use of a histopathological classification of cancers deriving from a particular organ is generally very satisfactory, nevertheless there are large numbers of unclassified tumours which it should be possible to define by their functional activities and in particular by their production of tumour markers.

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Nuclear Labelling of Leukaemic Blast Cells with Tritiated Thymidine Triphosphate after Daunomycin*

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Abstract—*The incorporation of ^{3}H-thymidine-5-triphosphate (^{3}H-TTP) into nuclei of leukaemic blast cells (LBC) was studied. Such labelling presupposes that the LBC contain replicative DNA polymerase and primer-template DNA. Besides, various other cytokinetic parameters were followed. In 8 cases of acute myeloid leukaemia the ^{3}H-TTP labelling index (^{3}H-TTP LI) was about three to ten times higher than the fraction in DNA synthesis. After a single i.v. bolus injection of daunomycin (DMC) an initial decrease was usually apparent after 6 hr. The nadir was reached at widely different intervals (6–56 hr) after DMC. A late rise in the ^{3}H-TTP LI took place and reached pretreatment values except in 2 instances. The ^{3}H-TTP LI in the blood was definitely lower than in the marrow. In 1 patient the ^{3}H-TTP LI of blood LBC was studied sequentially and closely followed the ^{3}H-TTP LI in the marrow. The present method cannot distinguish whether the action of DMC is on the primer-template DNA or on the activity of DNA polymerase. The data suggest that a high fraction of ^{3}H-TTP labelled cells is necessary for a normal flux into S. A moderate decrease in ^{3}H-TDR LI was seen late, i.e., 24–96 hr after DMC.*

INTRODUCTION

DURING the last years daunomycin has proved to be an effective drug in the treatment of acute leukaemia [1–3].

Daunomycin is known to inhibit DNA and RNA synthesis [4–7] by forming complexes with double stranded DNA [8, 9]. An inhibitory effect on RNA and DNA polymerase is also observed in cell-free systems [7]. Thus, Zunino *et al.* [10] demonstrated an inhibitory effect on the DNA-directed activities of DNA polymerase from murine sarcoma virus, rat liver, *Escherichia coli* and *Micrococcus luteus*. Inhibition of DNA polymerase by DMC has also been shown by other authors [11].

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Previous studies of the effect of DMC on leukaemic blast cells in human acute leukaemia have demonstrated a general direct killing effect on leukaemic cells [12–14] and an inhibiting action on the progression of cells through G_2 phase [12, 14]. Besides, long-lasting depressions of mitotic index (MI), ^{3}H-TDR LI and leucine incorporation by the leukaemic blast cells has been observed [14].

The purpose of the present paper was to study the effect of a single dose of DMC on the nuclear ^{3}H-TTP labelling of single LBC. As will be discussed later, the ^{3}H-TTP LI depends on cell content of nuclear DNA polymerase activity and primer-template DNA. At the same time, various other cytokinetic parameters were followed.

MATERIAL AND METHODS

Eight adult patients with acute myeloid leukaemia (AML) were studied. All patients, except 1, were previously untreated. Pertinent data of the patients are given in Table 1. Bone marrow (BM) and blood (B) samples were obtained at the time of diagnosis before DMC

Table 1. *Clinical and therapeutical data on patients. All patients were previously untreated except patient ALN who received* 20 *mg prednisone daily before and during the study period*

Patient	Sex/Age (yr)	BW	Treatment during study
AJE	M 65	68·8	DMC 105 mg after first BM
NMS	M 53	73·0	DMC 110 mg after first BM
AS	F 70	56·4	DMC 100 mg after first BM
ML	M 57	52·0	DMC 100 mg after first BM
HP	M 61	70·8	DMC 100 mg after first BM
ALN	M 66	58·0	DMC 100 mg after first BM, repeated after 36·5 hr
MO	M 77	61·0	DMC 100 mg after first BM
KM	F 74	73·4	DMC 100 mg after first BM, repeated after 24 hr

and at various intervals after DMC which was given as a single i.v. bolus injection as indicated in Table 2.

Smears of BM and B were performed without delay directly and stained with May–Grünwald–Giemsa. The percentage of blast cells in mitosis from late prophase to telophase (mitotic index, MI) was determined from a count of 3000–9000 blast cells in each sample. The total number of blast cells per μl of venous blood was determined from blood leukocyte counts and a differential count of 200 cells.

Part of the sample was incubated with ^{3}H-TDR (specific activity 2·0 Ci/mM, final concentration 1 μCi/ml BM or B) for 1 hr at 37°C. The percentage of ^{3}H-TDR labelled cells (^{3}H-TDR LI) was determined from autoradiographs (Kodak NTB-2, exposure time 7 days), counting at least 1000 cells.

Part of the remaining aliquot was smeared without delay on rinsed glass slides and fixed as described by Darzynkiewicz [15]. The incubation procedure was done as reported by Nelson and Schiffer [16] with a few modifications. Incubation chambers were made by attaching 24 mm glass rings to the slides. The incubation mixture consisted of 20 mM Tris ph = 7·8, 0·135 mM of 2′-deoxyadenosine-5′-triphosphate (dATP), 2′-deoxycytidine-5′-triphosphate (dCTP) and 2′-deoxyguanosine-5′-triphosphate (dGTP) respectively, and 5 mmol $MgCl_2$; final volume in each chamber was 0·5 ml; to this was added 10 μCi ^{3}H-TTP (Schwarz/Mann, Orangeburg, New York, USA, specific activity 40 Ci/mmole). Ficoll was added to the incubation mixture to make a concentration of 40 g/100 ml. The slides were incubated for 1 hr at 37°C. After fixation with formaldehyde, autoradiograms were done using Kodak NTB-2 liquid emulsion with an exposure time of two weeks. One thousand-five hundred to 3000 cell *nuclei* were counted to determine the percentage of nuclei labelled with ^{3}H-TTP (^{3}H-TTP labelling index = ^{3}H-TTP LI).

Counting TTP-labelled cells is often more difficult than counting ^{3}H-TDR labelled cells because the former labelling method requires a certain damage of the cytoplasm which sometimes is lost altogether. Photomicrographs of well preserved and less well preserved cells are shown in Figs. 1 and 2. In the first case, labelled cells can be counted directly. In the second case, all cells are counted and a correction for non-blastic cells made from a differential count (400 cells) of the direct smears. It should be mentioned that in these cases, non-blastic cells represent a very definite minority so that the possible error introduced in the ^{3}H-TTP LI count anyhow would have been small in case no correction had been made. This is to emphasize that populationwise, MI, ^{3}H-TDR LI, and ^{3}H-TTP LI can be directly compared.

RESULTS

(1) *^{3}H-TTP labelling index of marrow and blood blast cells Table* 2

Pretreatment ^{3}H-TTP in BM varied from 22·5% to 96%. In all eight patients the ^{3}H-TTP LI exceeded the ^{3}H-TDR LI by a factor 2·4–8·8. Without exception a decrease in the ^{3}H-TTP LI of LBC was seen after DMC. An initial decrease was usually apparent after 6 hr (except in KM). The nadir was reached at widely different intervals (6–

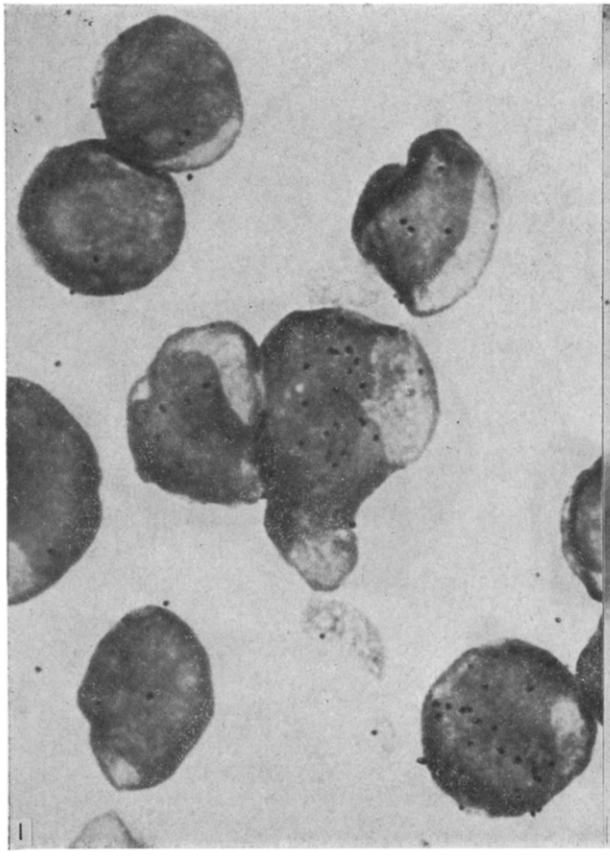

Fig. 1. ^{3}H-TTP *labelled nuclei of leukaemic blast cells in well preserved cells.*

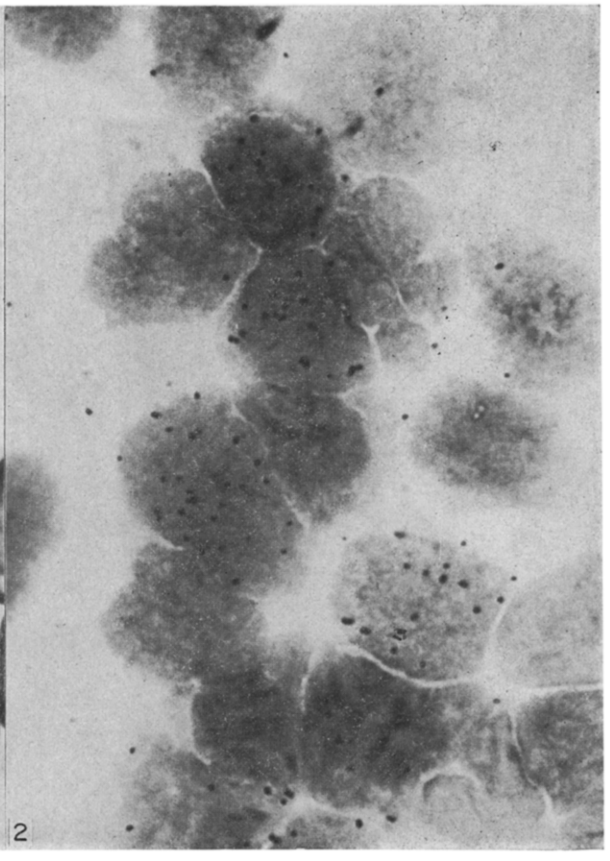

Fig. 2. ^{3}H-TTP *labelled nuclei of leukaemic blast cells in not well preserved cells.*

Table 2. Changes in cytokinetic parameters and ^{3}H-TTP labelling of leukaemic blast cells after daunomycin. The MI and ^{3}H-TDR LI values of AJE, NMS, and AS have been published previously [14]

Patient	Parameter	BM/B	Hours after DMC											
			0	6	8	12	24	32	36–36·5	48	56–60·5	69·5–72	82–85·5	94–96
	^{3}H-TTP LI	BM	57·8	31·1		19·5	13·6			13·0		16·9		31·0
	^{3}H-TDR LI	BM	6·0	7·6		8·3	9·8			7·9		8·1		4·9
AJE	MI	BM	0·20	0·00		0·05	0·25			0·25		0·23		0·25
	Blood blasts/μl	B	96	36		30	30			43		36		38
	^{3}H-TTP LI	BM	22·5	4·2		15·3	19·9			23·1				
	^{3}H-TDR LI	BM	7·4	6·1		4·1	3·2			4·1				
NMS	MI	BM	0·10	0·00		0·07	0·15			0·10				
	Blood blasts/μl	B	112	70		60	36			23				
	^{3}H-TTP LI	BM	55·7			37·2			38·2				57·8	
	^{3}H-TDR LI	BM	15·5			16·0			8·9				7·1	
AS	MI	BM	0·60			0·00			0·45				0·10	
	Blood blasts/μl	B	49,140			41,832			9,450				1,976	
	^{3}H-TTP LI	BM	31·6		23·8			10·3			26·3		21·0	
	^{3}H-TTP LI	B	22·1		4·7			0·2			13·7		6·2	
	^{3}H-TDR LI	BM	12·9		12·0			7·5			7·3		13·4	
ML	^{3}H-TDR LI	B	2·3		2·3			1·4			1·0		1·9	
	MI	BM	0·30		0·13			0·10			0·30		0·37	
	Blood blasts/μl	B	33,200		17,385			10,317			3,216		390	
	^{3}H-TTP LI	BM	31·0	21·8										
	^{3}H-TTP LI	B	14·2	11·4										
	^{3}H-TDR LI	BM	11·3	16·1										
HP	^{3}H-TDR LI	B	7·0	9·2										
	MI	BM	0·43	0·10										
	Blood blasts/μl	B	293,150	263,220										
	^{3}H-TTP LI	BM	96·0			62·4			38·5		63·8		100·0	
ALN	^{3}H-TDR LI	BM	14·3			11·7			12·4		8·4		9·6	
	MI	BM	1·27			0·45			0·70		0·55		0·83	
	^{3}H-TTP LI	BM	35·0				18·6				16·8	17·7		18·4
MO	^{3}H-TDR LI	BM	7·2				7·5				8·0	7·7		7·8
	MI	BM	0·77				0·45				0·72	0·92		0·7
	^{3}H-TTP LI	BM	26·9			39·4			14·7		27·0		62·6	
	^{3}H-TTP LI	B	10·3											
KM	^{3}H-TDR LI	BM	9·6			6·8			5·1		4·9		3·2	
	^{3}H-TDR LI	B	0·5											
	MI	BM	0·68			0·20			0·38		0·40		0·37	

56 hr) after DMC but in 6 of the patients the nadir was observed as late as 32–56 hr after DMC. After the nadir, a late rise in the ^{3}H-TTP LI took place and reached pretreatment values except in two instances. Values higher than the baseline was observed only in one patient (KM) who received DMC twice, namely 12 hr after the first dose and 60 hr after the second dose.

The pretreatment ^{3}H-TTP LI in blood varied from 10·3 to 22·1% in the three patients studied (Table 2). The ^{3}H-TTP LI in the blood was definitely lower than in the marrow. In one patient (ML), the ^{3}H-TTP LI of blood LBC was studied sequentially and closely followed the ^{3}H-TTP LI in the marrow although the initial decrease in the blood ^{3}H-TTP LI was somewhat more pronounced (Fig. 3).

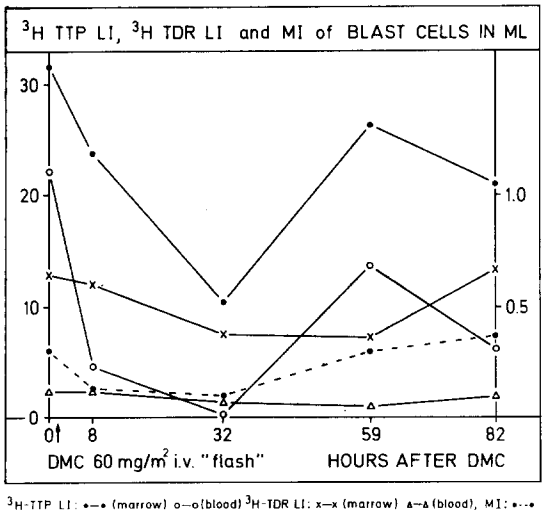

Fig. 3. An example of variations in ^{3}H-TTP LI, ^{3}H-TDR LI and MI of bone marrow and blood during treatment with daunomycin (Patient ML).

(2) *^{3}H-TDR labelling index and mitotic index Table 2*

Pretreatment ^{3}H-TDR LI in the BM varied from 6·0 to 15·5% and in the blood from 0·5 to 7·0% (Table 2). Except for patient MO, a moderate decrease in the ^{3}H-TDR LI of LBC was seen. The nadir was seen late, i.e., 24–96 hr after DMC. In four of the patients studied for a long period ($\geqq$ 84 hr), the ^{3}H-TDR LI was below pretreatment level 60–96 hr after DMC. It is interesting to note that the decrease in ^{3}H-TDR LI was relatively smaller than the drop in ^{3}H-TTP LI and took place later.

The initial MI value varied from 0·10 to 1·27%. Without exception an initial decrease in the MI was observed (6–24 hr). In four of the 7 patients studied for more than 24 hr, pretreatment values were reached 24–59 hr after DMC; in the remaining 3 patients the MI remained below baseline value 60–85, 5 hr after DMC.

DISCUSSION

1. *^{3}H-TTP LI before treatment*

The ^{3}H-TTP LI in LBC before treatment can be considered as a measure of the presence of nuclear DNA polymerase which is needed for replicative DNA synthesis. This is based on the following observations: Nelson and Schiffer [16] have demonstrated that with the present method one obtains in Sarcoma-180 mouse ascites tumors an estimate of nuclear DNA polymerase. It has been shown that both leukaemic blast cells [17] and lymphocytes [18] exhibit DNA repair after irradiation with u.v. light. However, non-treated lymphocytes do not exhibit observable repair synthesis and are negative when assayed for DNA polymerase by the TTP method. However, if the lymphocytes are stimulated to grow by phytohaemagglutinin they become ^{3}H-TTP positive [19]. Based on these studies it is reasonable to assume that any cell labelled with ^{3}H-TTP contains DNA polymerase for DNA-*replication* (and primer–template DNA). The range of the ^{3}H-TTP LI in this series is quite large, from 22·5% to 96·0%. Possibly, some of the lower figures may be underestimates since the assay is rather crude. However, in this case, one would expect to find a rather high fraction of cells with grains near the threshold. In the present study, a threshold of 5 grains has been used when counting in areas with very low background. By this approach there has been no doubt whether cells were labelled or not. We have tried to increase the threshold moderately to score a cell as "labelled" but this did not affect the LI markedly. The typical grain count of ^{3}H-TTP labelled cells is in the order of 10–20 grains. Moreover, extending exposure time up to 8 weeks did not increase the ^{3}H-TTP LI. Thus, the labelling with ^{3}H-TTP obtained in this study probably distinguishes quite well between cells with a high content and with no (or very low) content of DNA polymerase. Apart from this, the method is reproducible [20], and therefore can be used for comparative purposes which is the topic of this paper.

What then is the fate of cells which do not

label with ^{3}H-TTP? Probably, a fair fraction of these cells will not divide again but sooner or later die. It is very difficult to demonstrate death of leukaemic cells (and also normal haemopoietic cells). However, if one considers that the growth fraction of leukaemic cells is in the order of 15–35% and LBC have a cycle time of about 50–60 hr [21], one may compute that it would not last more than a few weeks before a patient with acute leukaemia would be transformed into a huge mass of leukaemic cells of the same weight as the patient. This obviously does not happen and is the best indication that there is considerable death among leukaemic cells. This is not to say that cells which will label with ^{3}H-TTP may not die. In fact, the maximum ^{3}H-TTP LI in this series was 96% and some of these cells must be expected to die. However, in general we believe that cells which do not label with ^{3}H-TTP are the most likely candidates for being end-cells.

The presence of DNA polymerase in the cell suggests that the cell has the capacity of DNA synthesis and cell division. This does not imply any particular model of the growth of leukaemic cells: the labelling indices observed here would be compatible both with a stem cell growth pattern, a stem cell growth pattern connected to one multiplicative compartment, or a stem cell compartment connected to subsequent serially connected multiplicative leukaemic cell compartments. It is interesting to note that the DNA polymerase containing cells are about three to ten-fold more frequent than cells in S-phase as measured by the ^{3}H-TDR LI. This demonstrates that the nuclear DNA polymerase is present not only during DNA synthesis but also in a large fraction of non-S-cells. Thus, DNA polymerase must be present in a large fraction of G_1 cells. Whether it is present in G_0 cells or not is a moot point and hard to answer because G_0 cells have not yet been formally identified in a human leukaemic cell population. However, if G_0 cells exist, there is a fair chance that they may label with ^{3}H-TTP. Our reason for believing this is a recent observation which indicates that liver cell nuclei all contain nuclear DNA polymerase [22], while it is well known that liver cells are very rarely in DNA synthesis or in mitosis unless the liver is damaged [23, 24].

2. *^{3}H-TTP labelling index after daunomycin*

As shown in Table 2 there was a definite effect of DMC on the fraction of cell nuclei labelled with ^{3}H-TTP. The response was variable but a definite alteration was observed in all patients. The general trend is an initial decrease in the fraction of ^{3}H-TTP labelled LBC by 6–12 hr; this may be followed by a further decline; the nadir is reached between 6 and 56 hr, and in some patients the ^{3}H-TTP LI returned to pretreatment values within the study period lasting up to 96 hr. In patient ML (Fig. 3) where both bone marrow and blood were studied, the pattern observed in the blood was equal to the changes in the bone marrow. It may be noted, in Table 2 and from Fig. 3, that the percentage of LBC labelled with ^{3}H-TTP in some instances came close to the number of cells in S-phase; however, the ^{3}H-TTP LI never got significantly below the ^{3}H-TDR LI. The effect of DMC on the fraction of cells in DNA synthesis as indicated by the ^{3}H-TDR LI was moderate and occurred late, as also described previously [14]. It holds true for all cases that the decrease in ^{3}H-TTP LI was considerably larger than the variations in the ^{3}H-TDR LI; even if the fraction of cells in DNA synthesis had fallen to zero (which did not occur, as indicated by the ^{3}H-TDR LI) such a decrease could numerically far from account for the fall in ^{3}H-TTP LI. The presence of thymidine-labelled and mitotic cells show that there still was a certain flux through S and M. However, the flux might be more impeded than the actual figures indicate, namely if the duration of S and M were prolonged due to damage inflicted upon the cells by DMC (e.g. it may be noted that mitotic time is prolonged after intensive treatment with cytosine arabinoside [25].

What is the explanation of the marked decline in the ^{3}H-TTP LI after DMC? There are two possibilities: (1) that DMC acts on the primer-template DNA of the LBC, and (2) an effect on the DNA polymerase activity of the LBC. From the data obtained both possibilities remain open. If DMC acts primarily on the enzyme it may be that the enzyme activity per cell exposed to DMC is reduced to the same extent in all cells. In this way many cells will fall below the grain count threshold to consider a cell "labelled". Another possibility would be that at least a fraction of cells containing DNA polymerase are more sensitive to the drug than cells with no detectable DNA polymerase. This cannot be decided. On the other hand, the data would be compatible with the notion that TTP-positive cells in late G_1 and S may be rather resistant, at least as judged from the initial stability of the ^{3}H-TDR LI, which suggests that for some time after DMC, cell flux from G_1 to S may not be particularly impaired. At later time intervals, the ^{3}H-TTP

LI rises whereas the ^{3}H-TDR LI tends to decrease. From this one may speculate that a rather large pool of DNA polymerase and primer-template DNA containing non-S cells is necessary to assure a constant influx of LBC into S-phase; in this respects, cells in late G_1 may be particularly important. As shown, a sudden decrease in the ^{3}H-TTP LI does not affect the ^{3}H-TDR LI immediately but apparently impairs flux into S at a later stage (Table 2). Such a delayed action on the flux into S may also explain that the ^{3}H-TTP LI rises later in the study period (in early G_1 cells?) without a simultaneous increase of the ^{3}H-TDR LI.

The late rise in the ^{3}H-TTP LI may either stem from originally TTP-negative cells, or from TTP-positive cells which temporarily became negative after daunomycin. That such repair is possible in principle is demonstrated by patient ALN who before DMC had a ^{3}H-TTP LI of 96% which dropped to 38·5% but later reached 100%. Here, at least, no visible ^{3}H-TTP LI negative cells could account for the full recovery of the ^{3}H-TTP LI 85·5 hr after DMC.

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Combination Chemo-Immunotherapy with Adriamycin in Experimental Tumor Systems*

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Abstract—*The possibility of integrating adriamycin (Adria) in combined chemo-immunotherapeutic treatments with non-specific immunomodulators was investigated. With an appropriate choice of treatment conditions clear synergistic effects were observed in the L1210 Ha leukemia when Adria was followed by treatment with* Corynebacterium parvum (C. parvum), *BCG and levamisole; however the Adria-*C. parvum *combination was found to be the most active.* C. parvum *appeared additionally to exert better antitumoral activity in this system when associated with Adria than with other antitumorals given in equiactive doses. The high therapeutic activity of the Adria-*C. parvum *combination was confirmed in three other leukemia systems (P388, LSTRA, L1210 Cr) even in conditions where chemotherapy with Adria alone was ineffective. Clear beneficial effects were additionally seen with this combination in the solid spontaneously metastatizing Lewis lung carcinoma, a system in which the triple therapeutic approach surgery-Adria-*C. parvum *was more effective than either of the binary combinations. The potential clinical implications of these results and the possible mechanisms at the basis of the synergism between Adria and* C. parvum *are discussed.*

INTRODUCTION

IN RECENT years a number of reports has accumulated indicating the effectiveness of immunotherapy in experimental tumors, and increasing evidence has appeared on its value in at least certain human neoplastic conditions [1, 2]. In the majority of clinical tumors, immunotherapy employed in the form of non specific immunomodulators such as BCG or *Corynebacterium parvum* (*C. parvum*) is preceded by chemotherapy [3–6]. Adriamycin (Adria) is an antitumoral drug on which much interest has lately focused because of its remarkable activity on a wide range of human malignancies [7]. Since a paucity of data appear to exist on the use of this agent in combined chemo-immunotherapeutic treatments, it was considered of interest to investigate in experimental systems the antitumoral potential of the association of this antibiotic with three immunomodulators (BCG, *C. parvum* and levamisole) chosen among those more frequently employed in the clinic [3–6, 8, 9].

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MATERIAL AND METHODS

Animals

Mice of both sexes of the following strains were used: C57Bl/6, Balb/c, $CD2F_1$ (Balb/c × DBA/2). The animals were obtained from Charles River Italy S.p.A. (Calco, Italy) and weighed 21 ± 1 g at the start of the experiments. Gross autopsy was performed on all animals and those found to be tumor-free 90 days after tumor transplant were considered cured.

Drugs

Adria and daunomycin (Dauno), obtained from Farmitalia (Milan, Italy), were freshly dissolved in sterile saline as done for 5-fluorouracil (5-FU) and cyclophosphamide (Cy) which were supplied by Dr. H. J. Wood

(DR & DT, NCI, Bethesda, Md.). Suspensions of formalin-killed *C. parvum* (lots PX 374 and 086 P, Wellcome Research Laboratories, Beckenham, England) were diluted with saline and injected i.v. in the dose of 0·7 mg/mouse. Fresh BCG preparations supplied by the Pasteur Institute (Paris, France) were injected i.v. in the dose of 1 mg/mouse; the standard dose of levamisole (Leva, Janssen Pharmaceutica, Beerse, Belgium) was 3 mg/kg i.p.

Tumors

The Lewis Lung (3LL) carcinoma was maintained and transplanted i.m. in syngeneic C57Bl/6 mice, the standard inoculum being 2×10^5 viable cells. Removal of the primary tumor was performed by disarticulation of the femural head of the tumor-bearing limb and measurement of metastatic dissemination to the lungs was carried out as previously described [10]. The L1210 and P388 leukemias were maintained by weekly i.p. passages in DBA/2 hosts, and transplanted in compatible $CD2F_1$ mice. Two different sublines of the L1210 leukemia were used in these experiments; the first, designated Ha, has previously been described as being immunogenic in compatible hosts [11]; the L1210 Cr subline was obtained from Dr. I. Wodinsky, A. D. Little Inc., Cambridge, Mass. and is currently employed in the antitumoral drugs screening program of the National Cancer Institute, N.I.H., Bethesda, Md. In immunosuppressed $CD2F_1$ hosts as well as *in vitro*, no differences were seen between the two sublines in their sensitivity to Adria and other antitumorals. The Moloney virus-induced LSTRA leukemia was maintained and transplanted i.p. in syngeneic Balb/c mice.

The number of tumor cells in the peritoneal cavity was determined as previously detailed [12] employing at least 5 animals per group. Results shown are net leukemic cell values subtracted of phagocytic-adherent cells whose proportion in the total ascitic population was determined as described [13], exploiting their capacity to adhere to glass surfaces and to phagocytize colloidal carbon particles.

Statistical analysis

Results presented are representative of at least three experiments performed employing 10 mice per experimental group. Statistical significance of the differences in survival was analysed by the Mann–Whitney U-test, by Fisher's exact test for the proportion of cures and by Duncan's new multiple range test for the evaluation of metastatic dissemination.

RESULTS

In preliminary experiments the chemotherapeutic activity of different i.v. doses of Adria given 1 day after implantation of 10^5 L1210 Ha leukemia cells was investigated. The greatest percentage of increases in lifespan (% ILS), without lethal toxicity, were seen with 10 mg/kg. This dose was therefore considered optimal in these conditions and employed for the subsequent tests.

Table 1 shows that single i.v. injections of 1 mg BCG on day 3, 6 or 8 after leukemia transplant did not significantly affect survival of the tumor-bearing mice; similarly ineffective were treatments on day 2, 4, 5 or 7 (data not shown). Significant increases in survival over that induced by Adria alone were seen when this BCG dose was administered 5 days after chemotherapy, this treatment also producing occasional cures (10–20% in 2 out of 6 experiments). This schedule was found to be the most effective in these conditions since the use of shorter (1, 2 or 3 days) or longer (6 or 7 days) intervals between Adria and BCG resulted in significantly lower % ILS or in results not different from those seen with Adria alone. Superimposable results were seen when the BCG dose employed was 2 mg.

Table 1. Antitumoral effects of adriamycin combined with BCG and levamisole on i.p. L1210 Ha leukemia

Day of administration of			
Adria	BCG	Leva	% ILS
+1	—	—	61
—	+3	—	0
—	+6	—	0
—	+8	—	0
+1	+3	—	65
+1	+6	—	110*
+1	+8	—	71
—	—	3–6	0
—	—	6–9	0
+1	—	3–6	74
+1	—	6–9	92*

10^5 L1210 Ha cells were transplanted i.p. in $CD2F_1$ mice on day 0; the Adria, BCG and Leva doses were 10 mg/kg i.v., 1 mg/mouse i.v. and 3 mg/kg i.p. respectively.

*$P < 0.05$ vs Adria alone.

When a 4 day course of Leva, *per se* not influencing survival, was given after an optimal Adria dose on day 1, a significant extra antileukemic effect was found when treatment with this adjuvant was initiated 5 days after chemotherapy (Table 1), the effectiveness of this

treatment being somewhat inferior to that seen with the most active Adria–BCG schedule since no cures were ever obtained. The use of other intervals (2 or 7 days) between Adria and the start of Leva treatment resulted in % ILS values not higher than seen with chemotherapy alone.

Table 2 shows that, whereas *C. parvum* alone was ineffective, significant schedule-dependent advantage was consistently found even with Adria dose (7·5 mg/kg) by itself giving only borderline ILS.

The kinetics of L1210 Ha leukemia cell growth and regression in the peritoneal cavity after chemo-immunotherapy with an optimal treatment of Adria–*C. parvum* is presented in Fig. 1. Treatment with the combined therapy resulted in a significant retardation of tumor

Table 2. Antitumoral effects of adriamycin combined with C. parvum *on i.p. L1210 Ha leukemia*

Day of administration of Adria	*C. parvum*	No. of L1210 Ha leukemia cells	% ILS	% Cures
+1	—	10^5	68	0
—	+3	10^5	18	0
—	+6	10^5	0	0
—	+9	10^5	0	0
+1	+3	10^5	100*	0
+1	+6	10^5	187†	80‡
+1	+9	10^5	75	0
+1	—	10^6	61	0
—	+6	10^6	2	0
+1	+6	10^6	75	60*
+1	—	10^7	55	0
—	+6	10^7	0	0
+1	+6	10^7	78*	10

L1210 Ha was transplanted i.p. in $CD2F_1$ mice on day 0; the Adria dose was 10 mg/kg i.v.

*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ vs Adria alone.

prolongations in survival were seen when this agent was combined with Adria. In fact, while 2 days intervals between the two treatments resulted in only a modest, although significant, extra increase in survival, a very clear synergism was found with an interval of 5 days as evidenced by the finding of 80% cures (range 70–90% in 10 experiments) of mice transplanted with 10^5 L1210 Ha cells. In contrast, results not better than found with Adria alone were observed when an 8 days interval was allowed between the treatments.

In view of the higher effectiveness of the Adria–*C. parvum* combination, this association was submitted to more extensive investigation in this and other neoplastic systems. In the L1210 Ha system, and employing the optimal 5-day-interval, this combination proved synergistic also when 10^6 or 10^7 leukemia cell inocula were used (Table 2).

When a range of Adria doses was given in combination with *C. parvum* (Table 3), the best results were obtained when the 10 mg/kg dose was employed; however, a clear therapeutic growth detectable already 2 days after *C. parvum* injection (i.e., day 8) so that on day 12 the peritoneal leukemia cell number of mice given chemotherapy alone was more than double in comparison to that present in hosts injected with both agents. At later times, in contrast to the additional increase in cell numbers observed in Adria-treated mice, a linearly progressive fall in leukemia cell counts occurred in animals submitted to combined treatment so that neoplastic cells were no longer detectable in the peritoneal cavity by day 19–20.

The potential of the Adria–*C. parvum* combination was confirmed in three other murine leukemia models as shown in Table 4. In addition to the trebling in survival given by the combination over that of Adria alone in the i.p. P388 leukemia, of special interest was the finding that this combination proved clearly effective in both the i.p. LSTRA and i.v. L1210 Cr leukemias, i.e., in systems where the highest tolerated Adria doses (7·5 and 10 mg/kg, respectively) were incapable of statistically prolonging survival.

Table 3. Antitumoral effects of different adriamycin doses in combination with C. parvum *on i.p. L1210 Ha leukemia*

Adria (mg/kg i.v.) day+1	*C. parvum* day+6	% ILS	% Cures	% Dead for toxicity*
2·5	—	7	0	0
2·5	+	10	0	0
5	—	15	0	0
5	+	40	0	0
7·5	—	32	0	0
7·5	+	63†	30‡	0
10	—	52	0	0
10	+	132†	80‡	0
15	—	122	0	20
15	+	173†	20	20
20	—	61	0	40
20	+	71	0	40
—	+	0	0	0

10^5 L1210 Ha leukemia cells were transplanted i.p. in $CD2F_1$ mice on day 0.
*Mice dead within 6 days of tumor transplant without evidence of leukemia at gross autopsy.
†$P < 0{\cdot}05$; ‡$P < 0{\cdot}01$ vs Adria alone.

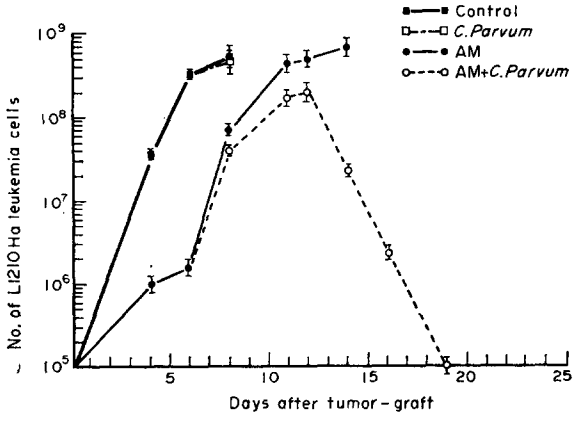

*Fig. 1. Kinetics of the leukemia cell numbers in the peritoneal cavity of mice given the Adria–*C. parvum *combination. Adria (10 mg/kg i.v.) was given on day 1 and* C. parvum *(0·7 mg/i.v.) on day 6 after the i.p. transplant of 10^5 L1210 Ha leukemia cells.*

The Adria–*C. parvum* combination was also investigated in the spontaneously metastasizing i.m. 3LL carcinoma, a model in which the effects of previous surgical removal of the primary tumor on the efficacy of subsequent chemo-immunotherapy with these agents could additionally be explored.

As shown in Table 5 by representative results of 3 such experiments, chemo-immunotherapy with Adria (10 mg/kg i.v. on day 11 when the primary tumor weight was 0·7–0·9 g) and *C. parvum* (0·7 mg on day 15) resulted in a better antineoplastic activity than either of the single treatments in terms of life-span increase, proportion of mice with metastases, weight of lung nodules. When surgery on day 9, which by itself significantly prolonged survival, was followed by Adria–*C. parvum*, this triple therapeutic approach was more effective than either of the binary combinations, both in terms of increase in median survival time and of the percentage of mice with lung metastases at sacrifice on day 23 (range 20–30% in 3 experiments). Moreover, surgery followed by this chemo-immunotherapeutic protocol resulted in a low (20–30%), but consistent, proportion of cured animals.

In view of the high activity of the Adria–*C. parvum* combination, it was considered of interest to examine in the L1210 Ha system, the effects of this immunomodulator when given after the injection of other antitumorals. When combined with *C. parvum*, doses of Cy equiactive with Adria in terms of %ILS produced a percentage of cures which, although remarkably high (between 20 and 50% in 3 experiments), was however significantly lower than that seen with Adria–*C. parvum* (Table 6). The 5-day-interval between Cy and the adjuvant appeared to be the most effective in these conditions since both shorter (3 days) and longer (7 days) intervals resulted in significantly lower % ILS values and proportions of cures (data not shown). As judged by the % ILS values, no added antitumoral effects were seen when *C. parvum* followed an

Table 4. Antitumoral effects of adriamycin combined with C. parvum *on P388, LSTRA and L1210 Cr leukemias*

Tumor	No. of cells on day 0	Adria Day	Adria Dose (mg/kg)	Adria Route	*C. parvum* Day	% ILS
P388	10^6 i.p.	+1	5	i.p.	–	50
	10^6 i.p.	+1	10	i.p.	–	65
	10^6 i.p.	—	—	—	+3	2
	10^6 i.p.	—	—	—	+6	2
	10^6 i.p.	+1	5	i.p.	+3	110*
	10^6 i.p.	+1	5	i.p.	+6	138*
	10^6 i.p.	+1	10	i.p.	+3	120*
	10^6 i.p.	+1	10	i.p.	+6	150*
LSTRA	10^4 i.p.	+1	5	i.v.	–	15
	10^4 i.p.	+1	7·5	i.v.	–	19
	10^4 i.p.	—	—	—	+6	0
	10^4 i.p.	+1	5	i.v.	+6	33†
	10^4 i.p.	+1	7·5	i.v.	+6	40‡
	10^5 i.p.	+1	7·5	i.v.	–	21
	10^5 i.p.	—	—	—	+6	0
	10^5 i.p.	+1	7·5	i.v.	+6	43†
L1210 Cr	10^3 i.v.	+1	10	i.v.	—	21
	10^3 i.v.	—	—	—	+6	0
	10^3 i.v.	+1	10	i.v.	+6	74*

*$P < 0.05$ vs Adria alone

†$P < 0.05$ vs control.

‡10% long term survivors.

*Table 5. Antitumoral effects of adriamycin–*C. parvum *on Lewis lung carcinoma*

Exp. group	Day of treatment	MdST* (days)	Primary tumor Wt (g ± S.E.)	% Mice with metastases	Wt of lung metastases (mg ± S.E.)
Control	–	24	8·7 ± 0·4	100	98·0 ± 16
Adria	11	28	5·4 ± 1·0†	80	23·1 ± 1.0†
C. parvum	15	29	5·9 ± 0·7†	100	42·2 ± 9·5†
Adria + *C. parvum*	11 15	35†	4·4 ± 0·5†	40‡[d]	9·4 ± 1·3‡
Surgery	9	30†	—	90	95·8 ± 44
Surgery + Adria	9 10	36†[a](1/10)	—	50	8·6 ± 0·3‡
Surgery + *C. parvum*	9 15	34†	—	70	58·0 ± 13
Surgery + Adria + *C. parvum*	9 11 15	46‡[bc](3/10)	—	20‡[cd]	3·0 ± 1·0‡

$2 \cdot 10^5$ 3LL cells were transplanted i.m. in C57Bl/6 mice (20 per experimental group) on day 0. Half of the animals were sacrificed on day 23 to evaluate primary tumor weight and metastases; survival was monitored on remaining 10 mice for 90 days. The Adria and *C. parvum* doses were 10 mg/kg i.v. and 0.7 mg/i.v. respectively.

*Median survival time. In parenthesis the number of long-term survivors. (Survival > 90 days).

†$P < 0.05$; ‡$P < 0.01$ vs control.

[a]$P < 0.05$ vs surgery; [b]$P < 0.05$ vs surgery + *C. parvum*; [c]$P < 0.05$ vs surgery + Adria; [d]$P < 0.05$ vs Adria alone.

Table 6. Antitumoral effects of adriamycin, 5-fluorouracil or cyclophosphamide when combined with C. parvum *on i.p. L1210 Ha leukemia in CD2F$_1$ mice*

Drug	Dose (mg/kg)	Day	% ILS	% Cures
Adria	10	+1	70	0
Cyclo	55	+1	63	0
5FU	200	+1	75	0
Adria + *C. parvum*	10 0·7 mg/mouse	+1 +6	120	90†
Cyclo + *C. parvum*	55 0·7 mg/mouse	+1 +6	140	40*‡
5FU + *C. parvum*	200 0·7 mg/mouse	+1 +6	67	0

10^5 L1210 Ha leukemia cells were transplanted on day 0.
*$P < 0.05$ vs control.
†$P < 0.001$ vs control.
‡$P < 0.05$ vs Adria–*C. parvum*.

injection of 200 mg/kg 5FU, i.e., a dose equiactive with 10 mg/kg Adria in terms of prolongation of survival. Preliminary experiments have also revealed no extra antitumoral activity when *C. parvum* followed by 5 days an injection of 10 mg/kg Dauno, a dose which shows maximal antileukemic activity in these conditions and is equiactive with 7·5 mg/kg Adria.

DISCUSSION

The first conclusion emerging from these results is that Adria, a drug at present widely employed in the treatment of various human malignancies [7], can successfully be associated with a series of non specific immunostimulators chosen among those in current larger clinical use. Clear synergistic effects were in fact observed in the L1210 Ha leukemia system when this chemotherapeutic agent was combined with each of the 3 adjuvants investigated, as evidenced by the obtaienmnt of increases in lifespan and/or cures in proportions not seen with chemotherapy employed alone, immunotherapy *per se*, in this model, being ineffective in the conditions investigated. An increased antitumoral effect of chemo-immunotherapy with Adria was not restricted to this system nor only to leukemias as shown by our findings with Adria–*C. parvum* in the 3LL tumor model. These data thus extend recent results of Houchens *et al.* [14] who have described enhanced therapeutic effects when Adria was given with *C. parvum*; the combination of this chemotherapeutic agent with other immunostimulants was, however, not examined in their study. More generally these findings are a further indication of the potential of chemo-immunotherapeutic approaches at least in animal systems and confirm a series of previous conclusions regarding the best conditions for the expression of this potential, such as the importance of the tumor burden at the start of therapy and of the time interval between the two treatments [15–20]. In this study, in which to conform with the more frequent clinical practice [3–6] immunotherapy was applied only after chemotherapy, an interval of 4–5 days between Adria and the administration of BCG, *C. parvum* or Leva was found to be optimal in the L1210 Ha system and was therefore used in our subsequent studies with Adria–*C. parvum* in other neoplasms. Although this schedule proved active, whether this interval was still the best in these additional systems was not investigated, nor conclusions can be advanced on whether the stringent interval dependency of the therapeutic efficacy of this combination observed in L1210 Ha also applied to the other models.

Significant antitumoral effects were seen with Adria–*C. parvum* in the LSTRA and i.v. L1210 Cr leukemia models in conditions where the highest tolerated doses of Adria alone did not produce significant increases in lifespan. If confirmed in other systems, this type of finding would suggest that to obtain significant therapeutic effects with an effective chemo-immunotherapeutic combination, a high tumor sensitivity to chemotherapy is not a strict requirement.

Considering that, in the conditions employed, i.e., metastatic dissemination already present at the time of surgery of the primary, the 3LL

tumor can be regarded as representative of a frequently encountered clinical occurrence, the significant effectiveness in this system of chemo-immunotherapy with an active combination such as Adria–*C. parvum* could have clinical implications, not only for the possible obtainment of better therapeutic results, but also for the possible use of lower doses of toxic chemotherapy to obtain the same antitumoral response. The latter aspect has here been directly demonstrated in the L1210 Ha system and is of special importance for a drug such as Adria for which cumulative cardiotoxicity is known to be limiting [7]. Also of possible clinical significance is the demonstration that the triple combination surgery–chemotherapy–immunotherapy was more effective than either of the binary combinations when effective chemoimmunotherapeutic combinations are employed. These results thus confirm previous data in this [21] and other systems [22] employing different cytoxic and immunostimulatory agents.

A further finding which may have direct practical implications for the most effective application of chemoimmunotherapy, consists in the marked variation in therapeutic activity seen when optimal Adria doses were combined with different immunoadjuvants. Adria–*C. parvum* was found in fact to be the most effective combination in the L1210 Ha leukemia when compared to optimal schedules of Adria in association with BCG or Leva. It is suggestive that in preliminary experiments Adria–*C. parvum* appeared more active than Adria–BCG also in the 3LL. It may be noted that also in a mouse mammary carcinoma system *C. parvum* proved more effective than BCG when given after chemotherapy with Cy [16]. Although not totally excludable, the possibility that the use of suboptimal doses of BCG and Leva could have played a role in this differential therapeutic efficacy in respect to *C. parvum*, seems unlikely. No greater effects were in fact seen when the BCG dose was doubled from 1 mg which has repeatedly been found as optimal in murine neoplastic systems [17]; an increase in the dose of Leva to 10 mg/kg was not followed by greater antitumoral activity when this agent was combined with Methyl-CCNU in the 3LL tumor [10]. Differences in the intrinsic immunostimulatory potency of the adjuvants tested could obviously represent an important factor in explaining this differential therapeutic efficacy; comparisons between these agents conducted in a representative series of experimental neoplasms are still lacking.

The fact that, despite the use of apparently chemotherapeutically equiactive doses, lower antitumoral activities were seen when *C. parvum* was combined with Cy, 5FU or Dauno than with Adria, leads to the conclusion that differences between these cytotoxic agents in activities other than direct tumor cell killing play a crucial role in this finding. Although no direct comparisons between these agents appear to exist, such a factor is presumably represented by their differential qualitative and/or quantitative capacity to interact with host immunity. In support of this contention is the now ample evidence that Dauno is significantly more immunosuppressive than Adria employed at equitoxic doses [13, 23, 24]. It would thus appear that an appropriate choice of both components of the chemo-immunotherapeutic association can be of great importance for rationalized treatments of this type. Studies are in progress to further analyse this aspect employing a larger number of such combinations in various experimental tumors.

The mechanisms at the basis of the observed synergism between Adria and *C. parvum* are a matter of discussion. Adria has been shown to relatively spare macrophages [13, 25] which are known to be a major target in the complex antitumoral effects of *C. parvum* [26] and are credited with an important role in antineoplastic resistance and especially metastasis formation [27, 28]. A complementary interaction of the 2 agents at this and possibly other levels could thus have been expected. No direct data on this point are given by this study; results to be reported elsewhere show that the *in vitro* non-specific cytoxic activity against tumor cells of macrophages recovered after treatment with Adria–*C. parvum* is as high as that seen after *C. parvum* alone. Both Leva [29] and especially BCG are known to stimulate macrophages [30], it is possible that quantitative differences in this capacity may be important in this context and/or that a complementation between Adria and *C. parvum* rests also at other levels. It is suggestive that in preliminary experiments it has been seen that the activity of the effector cells of antibody-dependent cellular cytotoxicity was as high in Adria–*C. parvum* treated mice as in those given the immunostimulant alone; these cells are known to be an important target of *C. parvum* immunostimulation [31].

Whatever the mechanisms, the clear activity of the Adria–*C. parvum* combination observed in this study appears to provide an experimental rationale to current clinical trials employing Adria with non specific immunostimulation; the preliminary results in this direction appear encouraging [32].

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Analysis of the Biotransformation of Benzo[a]pyrene in Human Fetal and Placental Tissues with High-Pressure Liquid Chromatography*

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Abstract—*The biotransformation of benzo[a]pyrene (BP) was studied* in vitro *in the presence of microsomal fractions of placental and fetal tissues from humans and monkeys* (Macaca nemestrina). *Metabolites formed in the incubation flasks were extracted and separated by means of high-pressure liquid chromatography utilizing a microparticulate column. In general, the formation of diols and quinones in fetal and placental homogenates was undetectable following* 15-*min incubations. The formation of phenolic metabolites, however, was easily measurable in fetal liver and lung and in the placenta but not in fetal spleen, kidney, pancreas or adrenal gland. The latter observation contrasted with high specific activities measured in the fetal adrenal gland with the fluorometric assay for aryl hydrocarbon hydroxylase activity. In placentas from cigarette smokers, relatively large quantities of an unidentified metabolite(s) appeared in metabolic profiles. This metabolite(s) did not co-chromatograph with any of the standard metabolites and the retention time was between those of the* 9,10- *and* 4,5-*diols. The same placental tissues catalyzed the covalent binding of BP to DNA and were far more active in this regard than any of the other fetal tissues investigated. The data indicated a correlation between metabolic profiles and capacity for catalyzing covalent binding to DNA for fetal/placental tissues.*

INTRODUCTION

IT NOW is widely accepted that environmental factors play a highly significant etiologic role as determinants of the incidence of malignant tumors in human populations. Most authorities estimate that such environmental factors are responsible for 70–90% of all malignancies and that chemicals constitute the major source of environmental carcinogens [1–3]. Tissue monooxygenases appear to play a key role in the initiation of chemically-induced tumors since they catalyze the conversion of inactive procarcinogens to highly reactive electrophilic intermediates capable of binding covalently to DNA and producing mutations in bacterial cells [4]. The oxidized intermediates presumably are the proximate or ultimate carcinogenic species.

The occurrence of vaginal clear-cell adenocarcinomas in human females (aged 7–29 years) whose mothers had ingested quantities of diethylstilbestrol during pregnancy [5–7] has focused attention on observations that chemicals can initiate tumors transplacentally. Investigations indicating that polycyclic aromatic hydrocarbons (PAH[4]) are capable of eliciting tumors transplacentally in rodents [8–10] create concern as to whether these chemicals could produce the same effects in humans, especially in view of recent reports of mixed-function oxidations of PAH in fetal tissues of humans and sub-human primates [11–13]. The high susceptibility of rapidly differentiating or developing tissues to initiation of tumorigenesis by chemicals [14] likewise focuses attention upon these problems. In the

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case of procarcinogens it may be expected that increased susceptibility of rapidly differentiating tissues to initiation of tumors could be compensated partially by low levels of monooxygenases in the same tissues. On theoretical grounds, however, the incidence of tumor initiation should be related to tissue concentrations of the reactive intermediate(s). These concentrations, in turn, may depend upon the activities of several different enzymes present in the tissues. High-pressure liquid chromatography (HPLC) allows a more critical evaluation of the overall metabolism of PAH in fetal tissues than previously utilized methods. Therefore, we have investigated a number of fetal and placental tissues of humans and pigtail monkeys with this technique. The rare opportunity to perform studies on fresh fetal tissues from the third trimester of human pregnancy provides considerable added interest and importance to this report.

MATERIAL AND METHODS

Chemicals

Glucose-6-phosphate, glucose 6-phosphate dehydrogenase (torula yeast), NADPH, NADH, calf thymus DNA (type I) and ATP were purchased from Sigma Chemical Company, St. Louis, MO. Benzo[a]pyrene (BP) was obtained from Eastman Chemical Co., Rochester, NY and recrystallized from benzene and methanol. N-2-fluorenylacetamide (FAA) was obtained from Mann Research Laboratories, New York, NY and recrystallized from methanol. BP-7,10-^{14}C (sp. act. 18·6 μCi/μmole) and BP-G-^{3}H (sp. act. 1600 μCi/μmole) were obtained from Amersham-Searle Corp., Arlington Heights, IL, and FAA-9-^{14}C (sp. act. 6·7 μCi/μmole from New England Nuclear Corp., Boston, MA. 1-, 3-, N-, 5- and 7-hydroxy-FAA were kindly provided as standards by Dr. E. K. Weisburger at the National Cancer Institute. The 3-hydroxy, 9-hydroxy, 3,6-quinone, 1,6-quinone, 6,12-quinone, 9,10-diol, 7,8-diol and 4,5-oxide metabolites of BP were obtained from Dr. H. V. Gelboin of the National Cancer Institute. The 4,5-diol, 4,5-quinone and 4,5-oxide also were synthesized in our laboratories according to methods described in the literature [15, 16]. All other reagents and chemicals utilized were of the highest purity commercially available.

Tissues

Human fetal and placental tissues (9–28 weeks gestation) were obtained from the Central Embryology Laboratory in the University Hospital, Seattle, WA. Tissues of one live-born fetus (at 28-week gestation) were obtained following a spontaneous abortion. Clinical details of this case are described below. Other human fetal tissues were obtained following routing terminations of pregnancies by hysterotomy, hysterectomy or dilatation and curettage. Gestational ages were between 9 and 16 weeks and were estimated from measurements of crownrump and foot lengths. Human placentas delivered at term were obtained from the delivery rooms of the University Hospital, University of Washington, and the Group Health Hospital, Seattle, WA. Fetal and placental pigtail monkey (*Macaca nemestrina*) tissues were obtained from the Regional Primate Center via the Central Embryology Laboratory. Two adult male rhesus monkeys were made available to us from the Department of Anesthesiology and results from those experiments are included for comparative purposes. One adult monkey was treated with 40 mg/kg of 3-methylcholanthrene via intraperitoneal injection 48 hr before sacrifice and removal of the various organs for assay. The fresh tissues were homogenized in 0·1 M potassium phosphate buffer (pH 7·4) in a polytron homogenizer. The other was treated with an equal volume of corn oil only. The homogenates were centrifuged at 8000 × *g* for 15 min and microsomal fractions were prepared by centrifuging the 8000 × *g* supernatant at 104,000 × *g* for 1 hr.

Clinical data from the spontaneous abortion of a 26-week, live-born fetus

A premature male caucasian infant born at 9.25 a.m. expired at 2.30 p.m. the same day. The infant weighed 860 g, was 13·25 in long, had no grossly observable abnormalities other than somewhat underdeveloped genitalia. The infant had been given 1 mg of phytonadione intramuscularly and 2 drops of silver nitrate solution (1%) in each eye following a facile spontaneous vaginal (vertex) delivery. One and five minute Apgar scores were both 2. The mother was 28 years of age, Rh negative (O), had one previous pregnancy in which a normal infant was delivered at term by Cesarean section and her nutritional status was rated as good. She had taken a prenatal vitamin preparation with iron during her pregnancy. She smoked 10–15 cigarettes per day, drank moderately and consumed 2–4 cups of coffee per day. After admission she received alcohol by i.v. infusion (2 courses) in an attempt to

delay parturition, and 100 mg of pentobarbital orally. She was known to be allergic to sulfa drugs. No other unusual history or clinical findings were noted with respect to either the mother or infant. Tissues were obtained from the Central Embryology Laboratory at 4.00 p.m. on the same day. Crown-rump and foot lengths were found to be 236 and 47 mm respectively. Liver, lungs, kidneys, pancreas, adrenal glands and spleen were placed on ice, taken to the laboratory and homogenized in ice-cold potassium phosphate buffer as described above. Unfortunately the placenta was not available for this study. Fractions were prepared in the evening, stored anaerobically overnight at −85°C and assayed the following morning. Tissues were maintained at 0–4°C throughout the procedure. Preliminary studies with monkey tissues indicated no significant differences in the enzymatic activities investigated between those incubated immediately and those stored overnight at −85°C.

Assays

Rates of 3-hydroxylation of BP (fluorometric assay) and of conversion of FAA to 7-, 5-, N-, 3-, and 1-hydroxylated metabolites in fetal and placental tissues were measured according to previously described procedures with equivalent incubation conditions [17]. Incubation flasks utilized in measurements of the metabolism of BP with HPLC contained BP-7,10-^{14}C (0·5 μCi/flask), 0·2 μmole BP, 120 μmole potassium phosphate buffer (pH 7·4), 3 μmole $MgCl_2$, 2·3 μmole NADPH, 10 μmole glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase and 0·5 ml of microsomal suspension in a total volume of 2·0 ml. The reactions were carried out at 37°C in total darkness in an atmosphere of 100% O_2 for 15 min. One gram of NaCl then was added, and the reaction mixture was extracted twice with 7 ml of ethylacetate: acetone (2.5: 1, v/v). The pooled extracts were evaporated to dryness under air in the dark. The metabolites were redissolved in 4·0 ml methanol which then was evaporated to near dryness and reconstituted to exactly 1·0 ml in methanol. Eight microliters were injected into a high-pressure liquid chromatograph (Micromeritics Model 7000–0111) for analyses. The metabolites were separated with a 2·1 mm × 8·0 cm permaphase ODS (DuPont) precolumn connected to a 4·1 mm × 25 cm Partisil ODS microparticulate column (Whatman, 10 μm) operated at ambient temperature with a constant flow rate of 1·0 ml/min and a 30–100% methanol–water gradient. Individual fractions were collected at 20 sec intervals directly into scintillation-counting vials beginning 3 min after the injection. Samples were collected for 30 min. Scintillation fluors (12 g omnifluor in 1 l of Triton X-100 and 2 l of toluene) were added and the samples were counted in a Packard Tri-Carb scintillation counter. Sufficient counts were accumulated to provide for 95% confidence intervals. Retention times of the radioactive metabolites were compared with the standard BP compounds listed above and are given in Table 1. Low quantities of impurities in the ^{14}C–BP obtained from Amersham-Searle did not co-chromatograph with any of the standard metabolites and appeared between the peaks containing BP and 3-hydroxy-BP. Heat-inactivated (100°C, 10 min) controls were run and the low values appearing in the resultant profiles were subtracted from the profiles of the test samples. Metabolites were tentatively identified on the basis of HPLC retention times. Standards were run several times during the course of these investigations in order to verify that retention times had not changed significantly. However, the very low activity of most of the tissues investigated as well as the unavailability of several known (hepatic) metabolites precluded further verification of peak identities.

Determinations of covalent binding of BP-^{3}H to DNA were based on the tissue-homogenate-mediated system originally described by Gelbon [18] and Grover and Sims [19]. Incubation flasks contained the following components in a final volume of 3 ml: 50 μmole sodium phosphate, pH 7·4, 100 μmole EDTA, 2 mg calf thymus DNA, 0·5 mg NADPH and 40 μg of BP-^{3}H in 25 μl ethanol (80 μCi). The reaction was started by the addition of hydro-

Table 1. *HPLC retention times for the standard compounds (BP and metabolites) utilized in the described experiments*

Compound	Retention Time	Fraction number
9,10-diol	11.30	35–36
4,5-diol	17.00	50–51
7,8-diol	18.05	53–54
1,6-quinone	19.00	56–57
3,6 and 4,5 quinones	20.40	61–62
6,12 quinone	22.00	64–65
9-OH	23.00	68–69
3-OH	24.00	71–72
Commercial contaminants*	24.30–25.30	74–77
BP	27.00–29.00	79–84

*Quantities of these radioactive contaminants, appearing in profiles of heat-inactivated (100°C, 10 min) tissue preparations, were subtracted from profiles of test samples.

carbon substrate and the mixture was incubated for 15 min at 37°C in the dark. The reaction was terminated by addition of 3 ml of a solution containing 2% SDS, 0·03 M NaCl, and 0·03 M NaCitrate at pH 7·0. The mixture was extracted twice with 1 volume of SDS-saturated phenol solution and the nucleic acids were precipitated from the aqueous phase by the addition of 3 vol of ice-cold 95% ethanol. The pellet was dissolved in H_2O and 0·3 ml of 2 M $MgCl_2$ was added, followed by 3 vol 95% ethanol. Subsequent centrifugation at 2000 × *g* for 20 min yielded a pellet that was washed twice more with 95% ethanol followed by one ether wash. The resulting nucleic acid pellet was hydrolyzed in 0·5 N PCA at 90°C for 20 min and aliquots taken for determination of DNA by the diphenylamine reaction [20] and radioactivity by liquid scintillation counting. The specific activities represent average values from 2 determinations with appropriate controls. Protein concentrations were estimated by the method of Lowry *et al.* [21].

RESULTS

An HPLC profile for the human fetal liver at 26 weeks gestation is presented in Fig. 1A. Essentially all of the metabolites appeared in fractions that co-eluted with 3-hydroxy- and 9-hydroxy-BP. Profiles from two livers at earlier stages of gestation were qualitatively similar but showed quantitatively lesser amounts of metabolites formed per unit protein in agreement with previous results obtained with fluorometric assays [22]. The profile for a fetal monkey liver is presented for comparison in Fig. 1B. This profile also exhibited some similarities to those obtained with human fetal livers but the ratio of quantities of metabolites in the 3-hydroxy-BP fraction to those in the 9-hydroxy-BP fraction were considerably lower in the fetal monkey liver (160 days gestation). By contrast, the profile of the MC-pretreated adult (rhesus) monkey liver exhibited large peaks coinciding with retention times of 3-hydroxy and 1,6-quinone standards, smaller peaks coinciding with retention times of 9-hydroxy, 6,12-quinone and 7,8-diol standards, and a large unidentified peak with a retention time between those of the 9,10 and 4,5-diols. No other metabolites were detectable. The adult monkey exhibited very low metabolism with most of the radioactivity coinciding with the 3-hydroxy standard small amounts coinciding with 9-hydroxy, 7,8-diol and the aforementioned unidentified peak also were detected.

An HPLC profile for human fetal adrenal glands (26-week gestation) is presented in Fig. 2A. In view of the high activity observed with the fluorometric assay (Table 2), the activity observed was surprisingly low. Nevertheless, the AHH activities obtained with the fluorometric assay were generally proportional to the capacities exhibited by the various tissues to catalyze the hydroxylation of FAA (Table 2). The metabolic profiles observed with fetal adrenal tissue were not markedly different from those obtained with heat-inactivated tissues except that the peak coinciding with 3-hydroxy-BP was slightly higher. Subsequent assays of monkey adrenal glands (fetal or adult) and a human adrenal gland from early gestation (14 weeks) likewise all exhibited very low activity by the HPLC method. The profiles obtained with human fetal kidney, spleen and pancreas (26 weeks gestation) were essentially identical to those

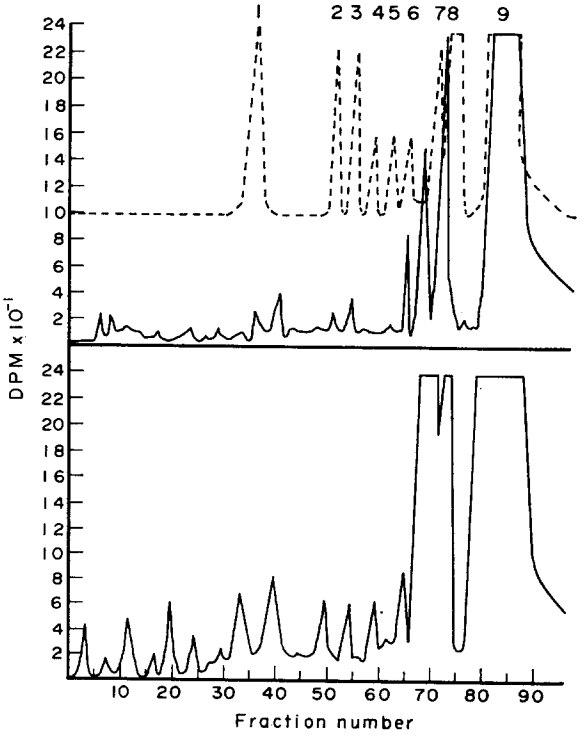

Fig. 1. *HPLC profiles of* ^{14}*C-BP metabolites formed in incubation flasks containing microsomal fractions of fetal livers of* (*upper*) *human* (26 *weeks gestation*) *and* (*lower*) *monkey* (22 *weeks gestation*). *Elution of standard BP metabolites is indicated by the numbers at the top of Fig.* 1*A:* 1. 9,10-*diol*, 2. 4,5-*diol*, 3. 7,8-*diol*, 4. 1,6-*quinone*, 5. 3,6- and 4,5-*quinones*, 6. 6.12-*quinone*, 7. 9-*hydroxy*, 8. 3-*hydroxy, and* 9. *BP. The elution pattern of the standard compounds is indicated by the broken line; elution of radioactive metabolites into respective fractions by the bars. Final protein concentrations in reaction flasks were* (*A*) 4·5 *mg/ml*, (*B*) 4·2 *mg/ml.*

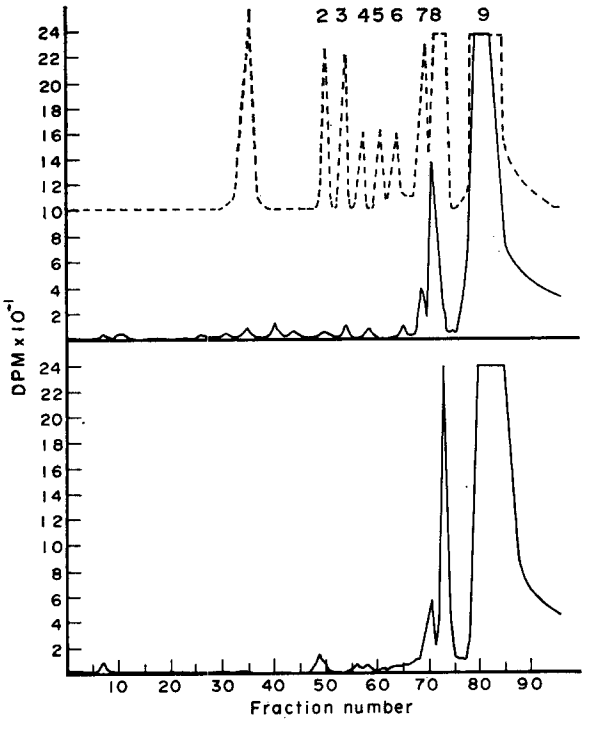

Fig. 2. HPLC profiles of ^{14}C-BP metabolites formed in incubation flasks containing microsomal fractions of human fetal (upper) adrenal glands and (lower) lungs. Both tissues were at 26 weeks gestation. Final protein concentrations in reaction flasks were (A) 0·6 mg/ml and (B) 2·1 mg/ml.

of the heat-inactivated controls. The HPLC profile obtained with fetal lung (Fig. 2B) tissue (26 weeks gestation) indicated considerably more activity than the adrenal glands with clearly distinguishable peaks coinciding with those of 3- and 9-hydroxy-BP and some very small peaks coinciding with the 6,12-quinone, 7,8-diol and 4,5-diol. Lungs from earlier gestation (12 and 14 weeks) however, exhibited profiles similar to those of the heat-inactivated controls. On the other hand, the profile obtained from the lung of the MC-pretreated adult rhesus monkey exhibited slightly less activity than the human fetal lung at 26 weeks gestation.

Typical profiles from human and monkey placentas obtained at term are illustrated in Fig. 3. The monkey placenta (Fig. 3A) consistently yielded profiles that were difficult to distinguish from heat-inactivated controls. Human placental microsomes yielded widely varying profiles—Fig. 3C illustrates a typical profile from a placenta with low to negligible AHH activity as measured with the fluorometric assay and Fig. 3B is a typical profile from a smoker's placenta that exhibited very high hydroxylase activity by the same assay. The largest peak coincided with the elution of the 3-hydroxy-BP. A relatively large peak coincided with the elution of the 1,6-quinone and another comparatively large peak eluted between the 9,10- and 4,5-diols as also was observed with the MC-pretreated adult (rhesus) monkey liver. Very small peaks coincided with elution of 6,12-quinone, 7,8-diol and 4,5-diol. The qualitative aspects of the profile were quite reproducible in placentas of 3 smokers but differed somewhat quantitatively as expected.

Other biochemical data from human fetal (26 weeks) and placental (term) tissues are given in Table 2. Interestingly, the data obtained with the fluorometric assay for measurements of rates of formation of 3-hydroxy-BP did not correlate well with data obtained with HPLC in some instances. This was particularly noticeable with the adrenal gland, pancreas and lung. However, the quantities of microsomes recovered from the adrenal

Table 2. Formation of hydroxylated products (pmole/mg protein/hr) from N-2-fluorenylacetamide and benzo[a]pyrene in microsomal fractions of human fetal (26 weeks) and placental (term) tissues as assayed with thin-layer chromatography and fluorescence spectroscopy respectively (see Methods)

Tissue	Origin	7-OH-FAA	5-OH-FAA	N-OH-FAA	3-OH-FAA	1-OH-FAA	3-OH-BP
Placenta 521	4·5	22·2	7·0	0·4	6·6	0·4	330
Placenta 522	47·4	394·2	25·2	5·2	64·6	0·4	50,420
Liver	29·1	55·6	1·2	27·9	10·8	19·2	1280
Lung	15·4	184·3	38·1	15·6	24·2	19·0	526
Kidney	6·1	47·9	1·3	4·7	18·6	8·4	870
Pancreas	17·8	194·7	13·5	27·4	49·6	25·6	1040
Adrenal	88·6	111·5	4·4	186·6	37·0	148·7	7920
Adult rat liver	1240	13,000	3790	1110	1600	311	39,700
Adult rhesus monkey liver (MC-pretreated)	5080	24,500	1960	840	2920	535	78,600
Adult rhesus monkey liver (control)	5607	6966	498	274	397	187	14,950

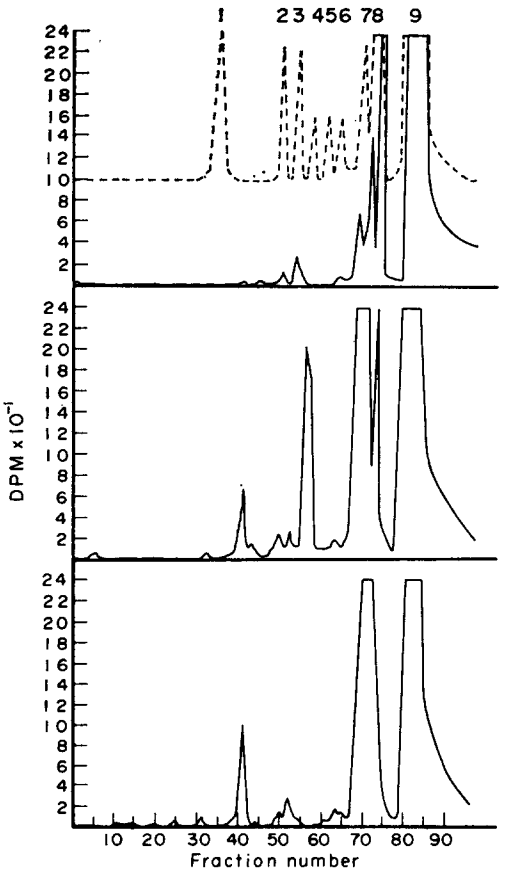

Fig. 3. HPLC profiles of ^{14}C-BP metabolites formed in incubation flasks containing microsomal fractions of placental homogenates at term from (upper) pigtail (middle) human (smoker) and (lower) human (nonsmoker). Final protein concentrations in reaction flasks were (A) 2·8 mg/ml, (B) 3·1 mg/ml and (C) 2·6 mg/ml.

gland and pancreas were quite low and, since the fluorometric assay is considerably more sensitive than the HPLC assay, it possibly reflects a more accurate measurement of the hydroxylating activity in the various tissues. Better correlations were observed with placenta, liver and kidney where much higher concentrations of microsomal proteins could be incubated.

The capacities of various human fetal (26-week gestation) and placental (term) tissues to catalyze the covalent binding of ^{3}H-BP to calf thymus DNA are given in Table 3. Of the tissues assayed, the placenta with the highest AHH activity likewise exhibited the greatest capacity to catalyze covalent binding to DNA. The lung also was comparatively active but the liver, kidney and spleen exhibited very low activities. The appearance of the metabolic profiles observed with the various tissues thus tended to correlate with the capacity of tissue enzymes to catalyze covalent binding to DNA. Unfortunately, there were insufficient microsomes from the adrenal gland and spleen to perform DNA-binding assays on those tissues.

Table 3. Covalent binding of ^{3}H-BP to DNA in the presence of microsomal fractions of human fetal (26 weeks) and placental (term) homogenates. Microsomes from the skin of methylcholanthrene pretreated (40 mg/kg, i.p. 48 hr before sacrifice) mice are included for comparison

Tissue	Specific Activity fmoles/μg DNA/mg protein/15 min
Placenta 521	18.7
—NADPH	8·6
Placenta 522	283·3
—NADPH	9·9
Liver	4·9
—NADPH	1·9
Lung	121·7
—NADPH	27·7
Kidney	Not detectable
Pancreas	Not detectable
Mouse skin	5·1
—NADPH	1·7

DISCUSSION

Interest in the utilization of high-pressure liquid chromatography for analyses of the metabolism of polynuclear aromatic hydrocarbons has increased markedly in recent years due to a number of advantages of the method, particularly when employed in conjunction with ^{14}C-labelled substrates. The older fluorometric assay is extremely sensitive and measures primarily the 3-hydroxylated metabolite (although other phenolic metabolites may contribute varying amounts of fluorescence [23]) but does not measure non-phenolic metabolites. A more recent assay procedure that employs ^{3}H-BP as substrate [24] is somewhat unsatisfactory due to the complications of the NIH shift [25]. The advantages of HPLC include simplicity, rapidity (chromatography can be completed in 30 min in our system without sacrificing resolution), good separation of phenols, quinones and diols and separations can be effected at ambient temperatures—the principal advantage over gas–liquid chromatography. Present techniques of HPLC, however, do not allow separation of all the known and possible metabolites although considerable improvement in the separation of phenolic metabolites now seems feasible through recycling [26]. The use of microparticulate columns in these studies enabled good separation of the 9- and 3-phenols and of the various standard quinones and diols. The quantities of 7- and 1-phenols in the peak containing the 3-phenol however, are unknown and will

require further investigation. Also, current techniques with HPLC do not provide the sensitivity of the fluorometric assay.

The present investigation illustrates the potential usefulness of HPLC in studying the metabolism of PAH in primate fetal tissues. Since by virtue of their high lipid solubility these ubiquitous environmental contaminants would be expected to pass readily from the maternal to the fetal blood, the desirability of obtaining these metabolic data is immediately obvious. This is particularly pertinent in view of observations that such chemicals act as carcinogens transplacentally in experimental animals [8–10] and that human fetal tissues contain the necessary enzymes for bioactivation via mixed-function oxidation [11–13].

The lack of correlation of enzymatic activities as assessed with various techniques for measuring BP metabolism also has been noted by other researchers [27–29]. In this study, the lack of correlation between activities measured with the fluorometric assay vs the HPLC assay were particularly noticeable in assays with fetal adrenal glands. Although much less adrenal microsomal protein was present in incubation flasks as compared with liver, lung, and placenta, this did not appear to entirely account for the apparent discrepancy. Specific activities calculated for the adrenal gland on the basis of the HPLC assay were much lower in relationship to the liver than when calculated on the basis of the fluorometric assay. Further research will be required to resolve this question.

Of interest was the observation that placental microsomes with high AHH activity (from smokers) were more active in catalyzing covalent binding to DNA than any of the other fetal tissues investigated. Previous studies [30] have shown that epoxide hydratase activities in human fetal placental microsomes are very low regardless of the hydroxylase activity detectable in the same preparation. Human fetal liver, lung, kidney and adrenal gland, however, exhibited activities comparable to those observed in adult rat livers. Thus, the data obtained with respect to DNA binding in this study are in agreement with that expected on theoretical grounds based upon the metabolic data. Since other studies [31, 32] have indicated that human placental AHH activity is localized in the endoplasmic reticulum of syncitial cells, it would be of interest to determine whether the incidence of choriocarcinoma in humans is in any way related to the exposure of women to polycyclic hydrocarbons.

A study of the biotransformation of BP in adult human tissues (liver and lymphocytes) with HPLC has been reported very recently [33]. It is of interest to note that the investigators likewise detected metabolites that did not co-chromatograph with any of the known standards. Whether or not the formation of these metabolites is peculiar to primate tissues remains an interesting question for future investigations.

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Serum Prolactin Concentrations Throughout the Menstrual Cycle of Normal Women and Patients with Recent Breast Cancer*

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Abstract—*Prolactin concentration has been estimated by radioimmunoassay of serum samples taken daily throughout the menstrual cycle of* 11 *patients who had undergone mastectomy for primary breast cancer and* 32 *normal women. Although there were no marked cyclical changes in prolactin level, concentrations were lowest in the follicular phase. Hence, comparison of normal and cancer subjects required detailed statistical analysis of results from comparable stages of the monthly cycle. Mid-cycle peaks of oestradiol and follicle stimulating hormone and the onset of menstrual bleeding were used as reference points. Prolactin concentrations were very similar in samples from normal and cancer groups, although at certain stages of the cycle some significant differences were found: on the fifth day preceding the mid-cycle oestradiol peak; during the follicular and periovulatory phases; and among the highest mid-cycle levels. At these stages of the cycle, samples from the breast cancer patients had a greater prolactin concentration than normal controls, although levels were within the normal range. The physiological relevance of these higher prolactin concentrations is uncertain and, in general, this detailed study clearly indicates little difference between serum levels of prolactin in normal women and in patients with breast cancer whose primary tumour had been removed more than three months previously.*

INTRODUCTION

ALTHOUGH the mammotrophic–lactogenic and luteolytic–luteotrophic effects of prolactin are well established in experimental animals [1, 2], the physiological role of prolactin during the human menstrual cycle is not so well understood. Apart from generally higher plasma levels in the luteal phase, and a possible increase near mid cycle, no consistent patterns of prolactin concentration have been found from daily blood sampling [3–8]. Indeed, such studies have emphasized the marked variations in prolactin concentration which occur not only between individuals but also from day to day within subjects. Furthermore, because cycles of differing lengths are usually aligned for periovulatory events, prolactin concentrations at the time of menstruation have been given much less attention [9].

The effects of prolactin on experimental mammary tumours are well documented, and their relevance to human breast cancer has been reviewed recently [10]. Whereas basal plasma prolactin concentrations were correlated with differing susceptibility to chemical induction of mammary tumours in 3 strains of female rats [11], a finding which also appears true for male rats [12], our studies of human breast cancer patients have shown similar basal prolactin levels at various stages of the disease and in control subjects [13, 14]. These and other studies [15–17] indicate that if there are abnormal prolactin concentrations in breast cancer patients then the differences will be small. However, elevated prolactin concentrations in breast cancer patients have been found

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using a heterologous radioimmunoassay [18] and also using a bioassay [19].

In this detailed study, basal prolactin concentrations throughout the menstrual cycle have been compared in normal women and in breast cancer patients, to examine the possibility that there may be differences at some stages of the cycle only. As it would be unethical to delay mastectomy for more than a month while collecting the daily blood samples, patients were studied at least three months after removal of the primary tumour, when they had resumed regular cycles. Such patients may still be considered to have breast cancer, though occult, since breast carcinoma is regarded by many as a systemic disease [20, 21].

MATERIAL AND METHODS

Subjects and samples

Samples of peripheral venous blood (10 ml) were obtained daily, or as often as possible, between 09.00 and 12.00 hr for at least one menstrual cycle. The blood was allowed to clot, centrifuged and serum removed to be stored at −20°C. The subjects were 32 normal women, and 11 women who had undergone mastectomy for primary carcinoma of the breast but had since resumed regular menstrual cycles. None of the subjects had a history of gynaecological disorders nor was taking drugs, hormone preparations or other agents known to affect prolactin, ovarian steroid or gonadotrophin levels.

Of the normal women, 11 were selected to be matched controls for the group of cancer patients on the basis of cycle length, age and completeness of sampling. The normal women, including matched controls, were placed into 3 subgroups by age for data analysis.

Measurement of prolactin, follicle stimulating hormone (FSH), oestradiol-17β and progesterone

Prolactin was measured by the radioimmunoassay established in these laboratories by Cole and Boyns [22]. Results are given as milliunits/ml M.R.C. Res. Std. A 71/222 where 1 mu = 50 ng prolactin by this assay system. Serum samples were assayed in duplicate, all those for an individual subject being together in one of the 8 assays that were required. Each cancer patient was paired with her matched control and their samples also included within the same assays. Displacement of 5% of bound iodinated prolactin was achieved by 0·04 mu/ml prolactin.

Measurements of FSH were limited to women over 40 years old. The radioimmunoassay of Groom *et al.* [23] was used and the results served to check whether subjects had normal cycles with a marked mid-cycle peak.

Oestradiol-17β and progesterone were determined by radioimmunoassays and the results for these subjects have been reported [24, 25].

Analysis of results

Prolactin concentrations were calculated from radioimmunoassay standard curves by the preferred equation of Taljedal and Wold [26]. Values of less than 0·01 were entered into subsequent calculations as 0·01 mu/ml.

Menstrual cycles varied greatly in length and so two preference points were used: the day of the mid-cycle peak of oestradiol-17β was designated Day 0, taking gonadotrophin and progesterone profiles into consideration; the day of appearance of menstrual bleeding was labelled M.

Prolactin concentrations were first examined to assess whether they met the assumptions inherent in parametric tests of statistical significance [27]. A logarithmic transformation was found necessary. Back-transformation yielded geometric means, but the logarithmic values were tested for significant differences.

As Student's *t*-test should be reserved for planned comparisons [27], for which the experimental design allowed relatively few, the Student–Newman–Keuls (SNK) procedure was applied to transformed daily prolactin values from the cancer, matched control, and normal 40s groups. The range of values in the cancer group was not wide enough to hold significant differences, but prolactin levels on days −5 and +1 in the other two groups lay beyond the maximum non-significant ranges at the 5% level. Planned comparisons were therefore made in the remaining groups using data from days −5 and +1.

The experimental design could support the use of Student's *t*-test for the following planned comparisons:

(a) test for homogeneity of normal subgroups and whether the cancer group is different.
(b) test highest periovulatory prolactin levels in cancer vs matched control groups.

Additionally, planned comparisons were made using samples taken during defined 5-day intervals which were evenly spaced over 4 consecutive weeks: days −1, 0, +1, 2, 3 (periovulatory phase); days −8 to −4 (fol-

licular phase); days +6 to +10 (luteal phase), and the period 2 days before and after Day M. By pooling the data in this way, the resulting prolactin levels should be less sensitive to misalignment of cycles. Values for the follicular phase were then compared with the periovulatory phase, and also the 5 days around menstruation, within all groups; the follicular phase prolactin in the cancer group was compared with matched controls and combined normals.

RESULTS

Mid-cycle peaks of oestradiol-17β or FSH could be located in 25 normal cycles and in all the 11 cycles from the patients with breast cancer. Two of the normal subjects (aged 53 and 46) were menopausal, having generally elevated FSH levels; critical samples were unavailable for a further 6 subjects and these were therefore omitted from detailed data analysis. One other cycle was rejected because of low FSH levels in an abnormally long luteal phase.

The remaining 24 normal cycles were placed in 3 age groups (Table 1). Distribution of ages within each group was essentially random, and cycle lengths were variable with no age-related trend. Table 1 also shows median value with range of both age and cycle length for the breast cancer group, matched control group and the combined normal group. The 30-year-old in the matched control group was chosen for her 22 day cycle to pair with the 23 day cycle of a 45-year-old cancer patient.

Prolactin concentrations did not follow a Gaussian distribution but approximated to a log-normal distribution. This is evident from Table 2 where for each group the median value from raw data is compared with the calculated arithmetic and geometric means. In a normal distribution, median and mean values coincide: in Table 2, the geometric mean is the closer to the median. Furthermore,

Table 1. *Allocation of subjects to groups, their age and the duration of their menstrual cycle*

Group	Age (years) Median	Range	Cycle length (days) Median	Range	Number of cycles
Normal 20s	25	22–29	30	26–32	8
Normal 30s	34	30–39	26	22–32	7
Normal 40s	44	40–49	27	25–32	9
Breast cancer	44	38–48	27	23–33	11
Matched control	41	30–49	27	22–32	11
Combined normal	35	22–49	28	22–32	24

Menstrual cycles of 23 normal women have been grouped by age and additionally by matching to the 11 cancer patients on the basis of cycle length and age. Note that 2 cycles were studied from one 22 year old subject.

Table 2. *Comparison of serum prolactin concentrations between groups of subjects using statistics of location and dispersion that are derived from raw data (median with 2·5–97·5 percentiles) and by arithmetic (mean, S.D.) or logarithmic (geometric mean ± 1·96 S.D. range) computation*

Group	Number of samples	Median value	2·5–97·5 percentiles	Arithmetic Mean	Arithmetic S.D.	Geometric Mean	± 1·96 S.D. range
Normal 20s	187	0·10	0·01–0·45	0·15	0·133	0·085	0·01–0·81
Normal 30s	147	0·09	0·01–0·33	0·12	0·090	0·083	0·01–0·53
Normal 40s	226	0·10	0·03–0·28	0·12	0·101	0·101	0·03–0·31
Breast cancer	292	0·11	0·02–0·44	0·15	0·112	0·114	0·02–0·53
Matched control	262	0·10	0·02–0·29	0,12	0·069	0·097	0·03–0·35
Combined normal	550	0·10	0·01–0·38	0·13	0·111	0·091	0·01–0·53

Serum prolactin concentrations in mu/ml MRC Res. Std. A 71/222 are given for menstrual cycles from subjects grouped as in Table 1. The median and 2·5–97·5 percentiles were found by inspection; arithmetic mean and standard deviation (S.D.) were calculated in the usual way; geometric mean ± 1·96 S.D. range are back transformations of the logarithmic mean ± 1·96 S.D., where 1·96 = t (approximately) at the 5% probability level for the appropriate degrees of freedom.

95% of raw data lie within the 2·5–97·5 percentiles by definition, and within ±1·96 S.D. of the mean of a normal distribution. These limits can be compared in Table 2 for each group of women, and it is obvious that a logarithmic transformation of prolactin values is reasonable.

Prolactin concentrations were similar in all groups of normal subjects and in the group of cancer patients (Table 2). The range of geometric mean prolactin for those individuals who were rejected from the normal group was 0·02–0·19, and this is similar to the ranges for normal subjects in their 20s, 30s and 40s or the range for breast cancer patients: 0·02–0·25; 0·02–0·21; 0·06–0·14; and 0·05–0·23 mu/ml respectively. There was no evidence for any trend in serum prolactin concentration with age (Fig. 1), and it can be seen that the geometric means for the cancer patients are within the range for the normal women.

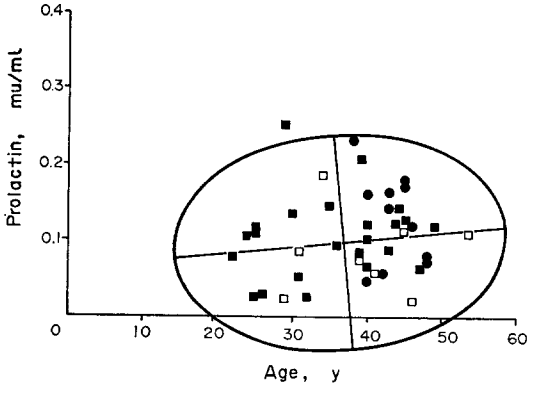

Fig. 1. Relationship between serum prolactin (geometric mean of daily samples over the menstrual cycle) and age. The ellipse was calculated to include 95% of data points from normal women. ■ Normal group; □ normal subjects whose prolactin concentrations were rejected from detailed analysis; ● breast cancer patients.

Daily prolactin concentrations for the follicular, periovulatory and luteal phases and the period of menstruation showed fluctuations of the geometric mean which were small compared with the range of the values for each day. Figure 2 illustrates this for the combined normal group. Nevertheless, prolactin levels were generally higher in the luteal phase than in the follicular phase, and an elevation at mid-cycle was present ($2P < 0{\cdot}025$ for day +1 vs −5 relative to the oestradiol-17β peak). A decline in serum prolactin occurred during the 2 days before onset of menstrual bleeding in 21 women out of 27 who were sampled on these days (significant at 1% level by a sign test).

When daily prolactin concentrations in breast cancer patients and matched controls were compared, a significant difference was found on day −5 in the follicular phase ($2P < 0{\cdot}01$) but not for day +1. A mid-cycle elevation of serum prolactin in the cancer group could not be shown ($2P > 0{\cdot}10$) whereas the significance level was 0·001 for day −5 vs +1 in the matched control group (Fig. 3). However, by using a paired comparison of the mean values shown in Fig. 3, Wilcoxon's matched-pairs signed-rank test indicated an overall difference between the 2 groups at the 2% probability level. This statistical method takes no account of the considerable range of prolactin concentrations associated with each mean (Fig. 2).

As the differences in mean prolactin levels within 5-day intervals were generally non-

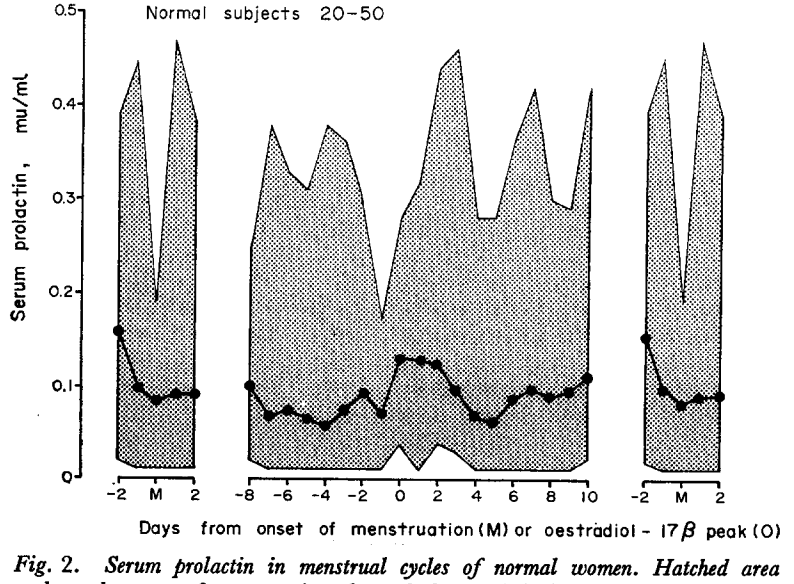

Fig. 2. Serum prolactin in menstrual cycles of normal women. Hatched area encloses the range of concentrations for each day, and the line denotes the geometric mean for 24 cycles.

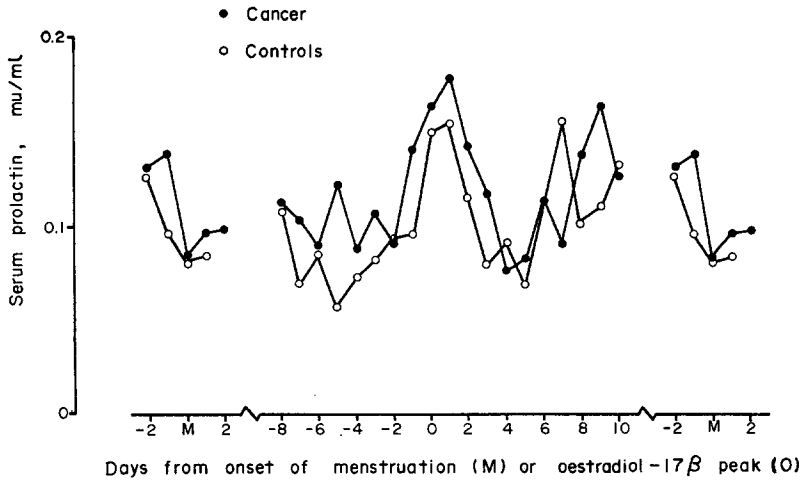

Fig. 3. Comparison of daily prolactin concentrations in breast cancer patients and matched controls. Values are geometric means for 11 *cancer patients* (●) *and* 11 *controls* (○).

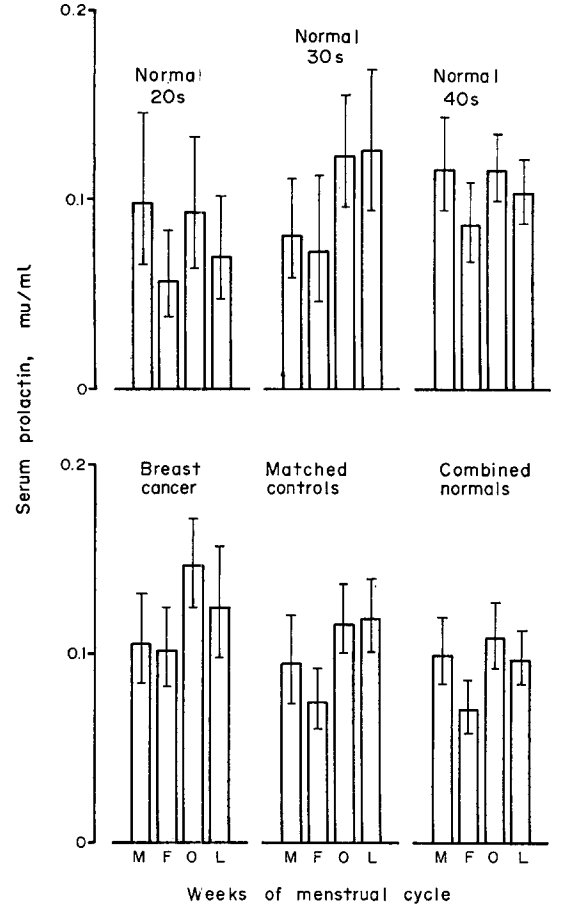

Fig. 4. Weekly patterns of serum prolactin in normal subjects, grouped according to age or selected as matched controls, and in breast cancer patients. Geometric mean with 95% *confidence limits are shown for prolactin concentrations over* 5*-day intervals in the weeks of menstruation* (M) *and ovulation* (O) *or within the follicular* (F) *and luteal* (L) *phases.*

significant, pooled values were calculated to represent the pattern of prolactin concentration week by week (Fig. 4). In each group, serum prolactin was lowest in the follicular phase and was significantly higher in the periovulatory phase ($2P < 0{\cdot}1$, 20s; $< 0{\cdot}05$, 30s; $< 0{\cdot}05$, 40s; $< 0{\cdot}01$, combined normal age groups; $< 0{\cdot}01$, cancer group; $< 0{\cdot}01$, matched controls). In all the groups of normal women, prolactin was higher in the week of menstruation than in the following week, significant at $2P < 0{\cdot}01$ for the combined group.

The weekly pattern of prolactin in serum from the cancer patients differs from normal (Fig. 4). Prolactin remained at the level of the menstrual phase during the follicular phase and was higher for the next 2 weeks. Consequently, there was a difference ($2P < 0{\cdot}05$) in the follicular phase serum prolactin concentration between 55 samples from 11 breast cancer patients and either 100 samples from 23 normal women or 49 from the matched controls. Similarly, these cancer patients have higher prolactin concentrations than normal in the periovulatory phase. In addition, by taking the highest values in the periovulatory phase, the geometric mean prolactin for the cancer patients (0·29) is greater than for the matched controls (0·20; $2P < 0{\cdot}025$).

DISCUSSION

Using samples taken throughout the menstrual cycle, only small differences could be found in serum prolactin concentrations between normal women and a group of patients that had undergone mastectomy for primary breast cancer. Indeed, to show any significant differences in prolactin levels, both the design

of the radioimmunoassays and the statistical treatment of results required very careful consideration. The physiological relevance of the differences is obviously uncertain.

The *a priori* selection of control subjects was intended to minimize possible sources of error due to interassay variation, age-related trends and bias arising from, for example, a prolonged follicular phase which would tend to lower an individual's mean prolactin level. In practice, however, the major variance components arose from within and between subjects. Hence, geometric mean prolactin values over the four 5-day intervals were within 2% of those given in Table 2 for which sets of results over the whole cycle were taken into consideration.

Several statistical treatments of the data were possible. Thus, prolactin concentrations could be normalized to reduce inter-subject variation and then tested for consistent cyclical changes. Alternatively, areas under the prolactin–time curve could be taken for the comparison of cancer and normal groups. Instead, the familiar Student's *t*-test has been applied to data which have undergone transformation in order that the assumptions of parametric significance testing were better met.

Prolactin concentrations approximated quite closely to a log-normal distribution, and a slight skewness may be accounted for by less than 0·01 mu/ml prolactin in the "prolactin-free" plasma that was used in constructing the radioimmunoassay standard curve. This skewness is a likely cause of the over-estimation of standard deviation terms which is indicated by the $\pm 1{\cdot}96$ S.D. limits being wider than the 2·5–97·5 percentiles (Table 2). The significance levels for differences between means are likely to be conservative estimates in consequence.

Daily serum prolactin concentrations were variable and there were no consistent patterns from one person to another during the menstrual cycle. This is in agreement with similar studies from other centres [3–7]. However, by pooling the results from the defined 5-day periods, a clear pattern of serum prolactin emerged. This pattern throughout the menstrual cycle was consistent insofar as follicular phase prolactin was lower than in the periovulatory or menstrual phases for the combined group of 24 normal cycles and in the subgroups of 8, 7 or 9 cycles by age, or the matched control cycles. However, the pattern for the breast cancer group differed because prolactin in the follicular phase remained at the level of the preceding week before increasing in the periovulatory and luteal phases (Fig. 4).

Comparison of patterns of serum prolactin week-by-week has advantages over the simple comparison of mean concentrations from one group of subjects with another. In addition to smoothing out daily fluctuations, assay and alignment errors should be minimal, and the errors due to the selection of patients and matched controls reduced. Thus, neither worry related to the mastectomy nor residual post-operative stress are likely to affect the pattern of prolactin levels over the month, whereas it could be argued that a single sample might well be affected.

The breast cancer patients for this study had undergone mastectomy for primary breast cancer and had since resumed normal cyclical activity. It is, of course, virtually impossible to obtain daily blood samples for a month from a woman with known or suspected breast cancer; it would be unethical to withhold treatment for this time, and hormone concentrations would be of doubtful value in a woman giving informed consent under such distressing circumstances.

The abnormal prolactin pattern in our patients may indicate some relationship between prolactin and carcinogenesis. The occurrence of significantly greater prolactin concentrations in the cancer patients during the periovulatory phase and the possible prolonged elevation at this stage (Fig. 3) certainly provide further evidence that the endocrinology of these women still differs if only minimally from the 23 normal subjects. Just as the disease persists in occult metastatic foci even after mastectomy [20, 21], so there may remain an abnormal prolactin balance in the breast cancer patients. However loss of breast tissue may itself affect prolactin secretion or utilization, although such a feedback mechanism from prolactin target tissue remains to be clarified. Mastectomy could result in neural stimulation of prolactin release, which might explain the generally elevated levels, but is unlikely to account for the observed difference in the weekly pattern.

Although differences in serum prolactin concentration were found, their interpretation requires care as it is probably unwise to generalize from a sample of only 11 premenopausal breast cancer patients. The differences between cancer and control levels are very small and their relationship to the disease is still uncertain.

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Effects of Prolactin and Suppression of Prolactin Secretion on Experimental Tumours of Lung and Muscle in Mice

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Abstract—*Prolactin is established as a growth factor for a number of animal mammary tumours. Yet prolactin at the appropriate time may inhibit mammary tumour development. We have tested the possibility that these actions may be systemic rather than organ related by investigating the effects of prolactin treatment and of suppression of prolactin secretion using 2 Br α-ergocryptine (bromocriptine) on two tumours of organs not thought at present to be prolactin dependent. Prolactin appeared to inhibit the growth but not the induction of urethane-induced pulmonary adenomas in mice and to reduce tumour growth and increase survival in mice injected with the Moloney sarcoma virus. Bromocriptine had no significant actions on pulmonary adenoma growth but when given over the period of urethane injection it dramatically reduced thymic uptake of tritiated thymidine. Bromocriptine reduced survival and increased tumour growth in animals treated with the Moloney virus: prolactin given with the bromocriptine prevented these effects.*

INTRODUCTION

THE MAMMARY gland is the best established target organ for prolactin. There are many examples in the literature of experimental mammary tumours whose growth is enhanced by elevation of plasma prolactin levels and slowed or abolished by reduction of prolactin levels [1–5]. However, there is also a considerable amount of evidence that if prolactin levels are elevated before and during the administration of a carcinogenic agent such as dimethylbenz-α-anthracene then the development and growth of mammary tumours may be prevented or slowed [6–10]. There is no adequate explanation for these conflicting findings. When prolactin stimulates tumour growth a proposed explanation is that the prolactin-dependent tumour tissue is able to outgrow the prolactin-dependent normal tissue. When prolactin inhibits tumour growth it is assumed that in some undefined way activation of normal mammary tissue may prevent tumour development.

One possible but uninvestigated explanation for these conflicting results lies outside the mammary gland. This is that some of the effects of prolactin on mammary tumour growth may be unrelated to the fact that the mammary gland is a target organ for prolactin. If prolactin had general effects on the inflammatory response or on the immune system then this in itself would have an effect on tumour growth which would interact with the target organ dependent effects. That this is not impossible is indicated by the inhibition of human lymphocyte responsiveness to phytohaemagglutinin produced by prolactin [11] and human placental lactogen [12].

We have therefore investigated in mice the effects of prolactin on two tumours of tissues not at present thought to be prolactin-dependent, the urethane-induced pulmonary adenoma [13] and the Moloney virus-induced rhabdomyosarcoma [14].

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MATERIAL AND METHODS

Urethane-induced pulmonary adenoma

Random bred mature male A2G mice were used. In each of two experiments four groups of 10 animals were used. The animals were

distributed among the groups in a way which ensured that the mean starting weight of each group was the same. The groups were treated daily as shown. All injections were given subcutaneously in the afternoon in a volume of 0·1 ml.

(1) Control: 0·9%.
(2) Ovine prolactin (Ferring, Malmo, Sweden): 50 μg.
(3) Ovine prolactin 250 μg.
(4) 2 Br α-ergocryptine (bromocriptine): 100 μg. Bromocriptine is a long acting dopamine agonist and a potent inhibitor of prolactin secretion [4, 5, 15].

The prolactin was not contaminated by LH, FSH, ACTH or TSH as shown by radioimmunoassay. Growth hormone contamination was less than 2%. It contained less than 10 ng/mg of arginine vasopressin (W. B. Malarkey, personal communication).

Urethane was given i.p. to all animals in a dose of 1 mg/g body weight. In the first experiment the various treatments began 2 weeks before the urethane and continued for 4 weeks afterwards. In the second experiment the treatment began 8 weeks after the urethane and continued for a further 8 weeks. Each experiment thus lasted for 16 weeks. One hour before death each animal was given 0·5 μCi/g of tritiated thymidine i.p. After death the thymus and the lungs were removed. The thymus was weighed and its radioactivity counted. The lungs were fixed in Carnoy's solution. Tumours on the surfaces of both lungs were counted and measured using a dissecting microscope. The sum of the products of the horizontal and vertical widths of each tumour gave the area index. From the left lung 5 μm thick paraffin sections were prepared for autoradiography using a stripping film technique. The labelling index in the tumours was estimated by counting the number of labelled nuclei at a ×1000 magnification in different fields that contained a total of 3000 labelled and unlabelled nuclei. The index was equal to the number of labelled nuclei counted/total number of nuclei scanned multiplied by 100.

All parameters in the treated groups were compared with the control groups using Student's *t*-test.

Moloney virus-induced rhabdomyosarcoma

The experiments were carried out in male Balb/c mice. At 25 days of age each animal was injected in the mid-left thigh with 0·2 ml of crude tumour homogenate (20% of tumour by weight in Earle's saline) kindly provided by Professor J. J. T. Owen. Tumour growth was monitored for the next 14 days by measurement on alternate days of the thigh width at the point of injection. Seven experimental groups of 10 mice and one control group of 20 mice were used and were given daily the treatments shown by subcutaneous injection. Each treatment began 3 days before the virus inoculation and was continued until the end of the experiment.

(1) 0·9% saline.
(2) 50 μg ovine prolactin.
(3) 250 μg ovine prolactin.
(4) 500 μg ovine prolactin.
(5) 250 μg bromocriptine.
(6) 500 μg bromocriptine.
(7) 50 μg prolactin + 250 μg bromocriptine.
(8) 500 μg prolactin + 500 μg bromocriptine.

For each animal, its thigh width on day 0 was taken as 100% and subsequent changes in thigh width were first expressed as percentages of this. The mean percentage change in the control group on each day was then estimated. The percentage change in each experimental animal was then expressed as a percentage of the mean percentage change in the control group. The results in each group were finally expressed as a mean percentage deviation from control, with the mean control value on each day being taken as 100%. The experimental groups were compared with the control groups by an analysis of variance.

RESULTS

Urethane-induced pulmonary adenoma

The results are summarised in Fig. 1. When the treatments were given over the period when the urethane was injected only two significant findings were observed but these were very striking. The thymus uptake of tritiated thymidine and the tumour labelling index were both dramatically reduced ($P < 0·001$) in the group of animals given bromocriptine. The weight changes in the various groups are shown in Table 1.

When the treatments were started 8 weeks after the urethane by which time the tumours would have been established and begun to grow the only obvious effects were a reduction in the number of visible tumours and the total area index of the tumours in both the prolactin-treated groups. The effect of the lower dose of prolactin was more marked than that of the

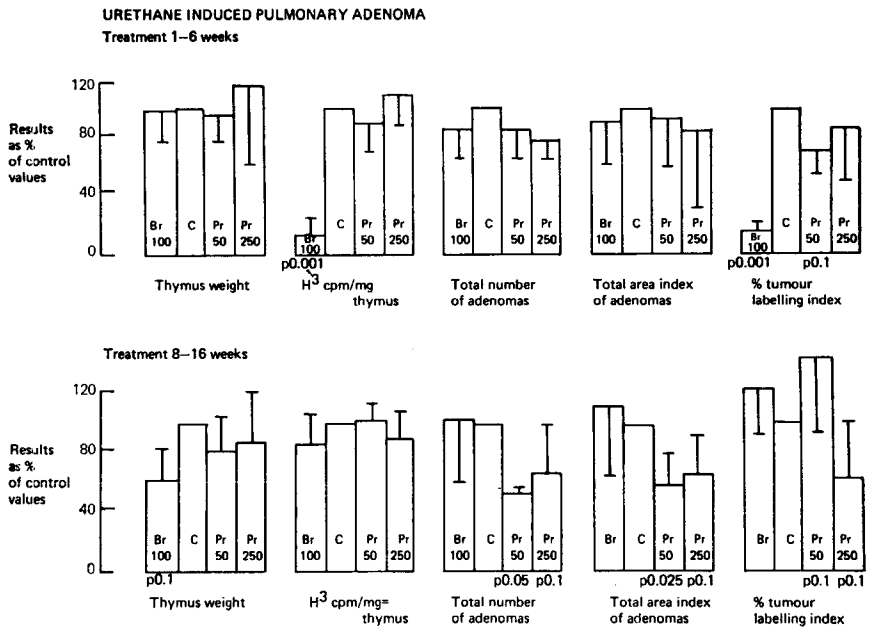

Fig. 1. *The effects of treatment with prolactin and bromocriptine on the development and growth of urethane-induced pulmonary adenomas in mice and on thymus weight and thymus uptake of tritiated thymidine. Detailed experimental protocols as described in the text. C, control: Br*100, *bromocriptine* 100 *μg/day: Pr* 50, *prolactin* 50 *μg/day: Pr* 250, *prolactin* 250 *μg/day. For each parameter the mean value in the control group was noted: each result in the other groups was then expressed as a percentage of this mean control value. The bars indicate the mean percentages of control* ± *S.E.M.*

Table 1. *Weights in g at* 16 *weeks in the various groups of animals given urethane. Initially the animals were distributed so that the mean weight in all the groups on starting the experiment was* 26·6 g. *All figures represent means* ± *standard deviation*

Animals treated from 0–6 weeks after receiving urethane		
Control		33·6 ± 2·30
Prolactin	50 μg/day	32·9 ± 3·11
Prolactin	250 μg/day	32·9 ± 4·63
Bromocriptine	100 μg/day	33·3 ± 2·29
Animals treated from 8–16 weeks after receiving urethane		
Control		32·6 ± 3·75
Prolactin	50 μg/day	32·3 ± 2·93
Prolactin	250 μg/day	33·0 ± 2·62
Bromocriptine	100 μg/day	33·5 ± 3·34

higher dose. The mean surface area of each tumour did not differ between the groups.

Moloney virus-induced rhabdomyosarcoma

The tumours developed at the site of inoculation approximately 5–7 days after virus administration, reached a maximum size by 9 days and thereafter regressed. The growth pattern in the control group is shown in Fig. 2a: mean thigh width on day 0 was 8·02 mm ±2·10 (S.D.) and on day 5, 13·86 mm ±2·53. In Figs. 2b and 3 the growth patterns in the other groups are expressed as deviations from the control. Differences between the groups were studied by analysis of variance.

The most striking results concerned survival at 14 days after injection of the virus (Table 2). One in twenty control animals, 0/30 prolactin treated animals and 10/20 bromocriptine treated animals died ($P < 0{\cdot}001$). The administration of exogenous prolactin largely prevented deaths associated with bromocriptine treatment: only 1/20 of the animals which received both treatments died.

With regard to tumour size, 50 μg/day prolactin consistently inhibited tumour growth ($P < 0{\cdot}005$). The two higher doses had inconsistent effects: tumour size was just significantly greater than control in the 250 μg/day group ($P < 0{\cdot}05$) from the 8th to the 12th day. Tumour size in the group which received 250 μg/day bromocriptine was significantly greater than control from the 6th to the 10th days ($P < 0{\cdot}05$) while results with the 500 μg/day group showed no consistent differences from control. When prolactin and bromocriptine were given together the tumour growth pattern was extremely close to that of the controls.

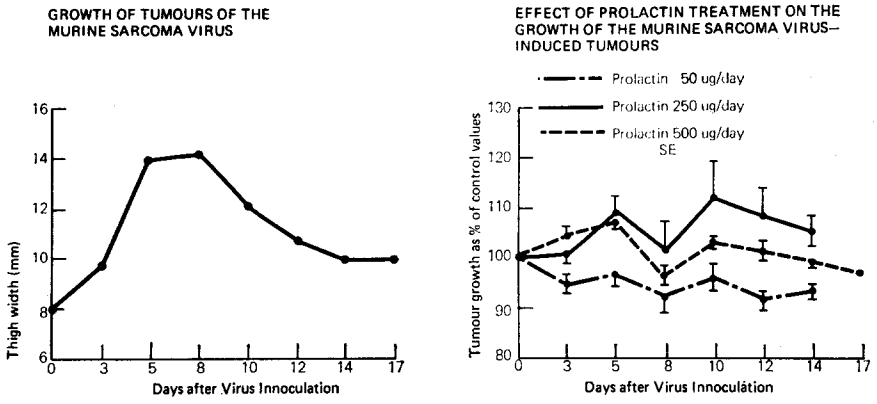

Fig. 2. *Left* (a). *The growth pattern of the murine sarcoma virus in the untreated control group. The mean thigh width in the control group on each day was taken as* 100%. *In the other illustrations, the effects of the various treatments are on each day expressed as percentages* ± *S.E.M. of the control value on the same day. Right* (b). *The effects of prolactin treatment alone at various concentrations on the growth pattern of the tumours.*

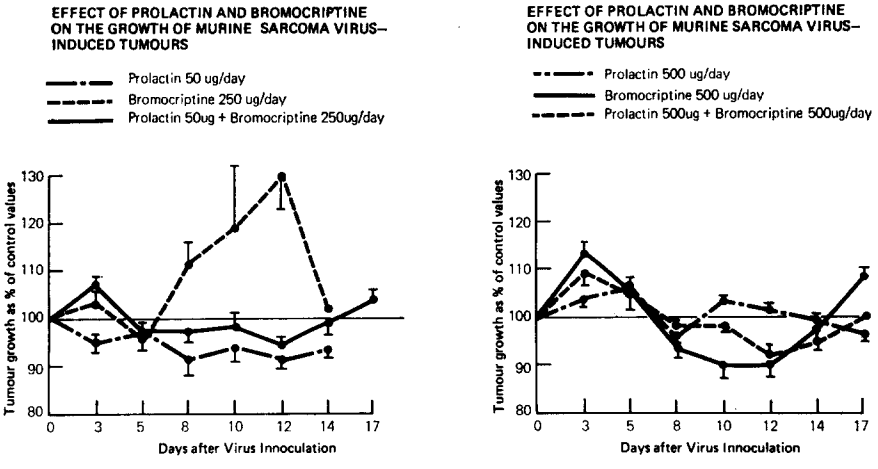

Fig. 3. *Left. Comparison of the effects of the lower doses of prolactin and of bromocriptine and of both given simultaneously on tumour growth. Right. Comparison of the effects of the higher doses of prolactin and of bromocriptine and of both given simultaneously on tumour growth.*

Table 2. *Survival data for animals injected with the Moloney sarcoma virus homogenate and treated in various ways*

	Total	Alive	Dead
Control	20	19	1
Prolactin (50, 250 or 500 μg/day)	30	30	0
Bromocriptine (250 or 500 μg/day)	20	10	10
Bromocriptine + prolactin	20	19	1

DISCUSSION

Urethane-induced pulmonary adenoma

This tumour was first described in 1943 [13] and has been the subject of many subsequent reports [16–21]. The urethane is believed to have both a direct local carcinogenic effect [22] and a systemic effect [23] in lowering the resistance of adenoma development.

The results of the present experiments show that none of the treatments given to cover the time at which urethane was administered had any effect on the tumour development at 18 weeks. This suggests that the treatments did not have any effect on the actual numbers of tumours induced and possibly did not have any important action on their growth in the early stages. The most striking finding in this part of the experiment was the dramatic reduction in thymus uptake given bromocriptine at the time of urethane administration. No such interference with thymidine uptake took place when the bromocriptine was given from 8–16 weeks.

We would be inclined to dismiss this as an unexplained artifact but for our finding that in mice of the Bar Harbor 129 strain bromocriptine has a similar profound action on thymidine uptake by the thymus [24]. It has been suggested that urethane may be toxic to the thymus [25] and this study indicates that in the absence of prolactin the toxic effect may be markedly enhanced. The Bar Harbor 129 mice seem to have some abnormality of thymus development and in this case again bromocriptine appeared to exaggerate this.

We have no satisfactory explanation for the dramatic decrease in tumour labelling index in the same group of animals which showed the sharp fall in thymidine uptake by the thymus. The labelling index theoretically should be a measure of the mitotic activity of the tumour. If this is so then tumours with a low labelling index should be smaller than others but there was no evidence that this was so. The same tumour size might be attained with a low labelling index if cell loss from the tumour were sharply reduced and this could be correlated with a failure of the immune response to the tumour. However there appeared to be little if any lymphocytic infiltration of the tumours in any group with no differences between the groups.

When the treatments were given from the 8th to 16th weeks prolactin clearly reduced the numbers of the visible adenomas. Since prolactin had no apparent effect on induction this must mean that some of the tumours stopped growing in the very early stages or disappeared altogether. The sizes of the individual tumours that were left as shown by the mean area indices were not significantly different from the sizes of the tumours in the other groups. This suggests that there may be two populations of tumours, one unaffected by prolactin and one susceptible to suppression in its presence. Variations in the antigens carried by the tumours might account for this.

Moloney virus-induced rhabdomyosarcoma

The tumours induced by inoculation of Moloney sarcoma virus into mouse muscle have been classified as rhabdomyosarcomas [14]. The nature of the sarcoma varies according to the animal strain inoculated, the age of the animals and the virus dose. Tumours induced in immunologically hyporesponsive hosts grow progressively and kill while those induced in more reactive hosts grow rapidly at first but then regress [26]. The regression is probably mediated by an immunological reaction against tumour specific transplantation antigens (TSTA) [27–29] which this tumour possesses. Reaction against TSTAs primarily depends on lymphocytes [30–33] but cytotoxic humoral antibodies may also be involved [34–36]. Two mechanisms of *in vitro* growth inhibition in the Moloney system have been suggested [37], the first non-specific and probably mediated by macrophages and the second specific and probably mediated by non-adherent cells. Macrophages, of course, could interact with lymphocytes and the armed macrophages and sensitized lymphocytes could recognize the TSTAs in tumour cells [38, 39].

Our experiments left no doubt that animals in which prolactin secretion was suppressed by bromocriptine were frequently killed by the Moloney virus inoculations. Bromocriptine is a long acting dopamine agonist [15] and could have many actions in the body apart from suppression of prolactin secretion. However, the relationship of the prolactin suppression to the deaths is strongly suggested by the reduction of deaths to one only out of the 20 animals which received exogenous prolactin in addition to the bromocriptine. Presumably actions of bromocriptine unrelated to prolactin suppression would have continued to be operative in this situation. If death due to the Moloney virus is related to hyporeactivity of the immune response then this may indicate that prolactin has a key role to play in the maintenance of a healthy immune system.

The lowest of the three doses of prolactin seemed to be the most effective in maintaining the animals in a healthy state and containing tumour growth. The higher doses may even have enhanced growth slightly. These observations are consistent with the effects of prolactin in other systems where repeatedly it has been observed that a low to moderate amount of prolactin has one effect which may be lost or even reversed when higher amounts are used [4, 5, 15].

GENERAL COMMENTS

These experiments are provocative in three main ways. First they are relevant to the interpretation of studies on the effects of prolactin on mammary tumours. Such studies to date have almost invariably been interpreted in the light of the concept that the mammary gland is a target organ for prolactin. Our findings using organs which at present are not thought to be prolactin target organs indicate that the

hormone can have a clear action in inhibiting tumour growth. The inhibition of mammary tumour growth by prolactin which has repeatedly been observed may in part therefore be dependent on the systemic effects of the hormone.

Second the experiments show that prolactin may have important actions on the growth of a variety of tumours. They therefore indicate that endocrine effects on tumour growth, even in tissues not thought to be hormone dependent, are worthy of further study. The fact that prolactin is now known to be present in both sexes and that in both its secretion rises dramatically in response to such stresses as emotional disturbance, exercise and surgery [40] may have both theoretical and practical implications in cancer treatment.

Third the experiments suggest that prolactin may have actions on the immune system. The effects of bromocriptine on the thymus uptake of labelled thymidine and on survival of the animals treated with the Moloney virus homogenate indicate that normal levels of circulating prolactin may be involved in the maintenance of the normal function of the immune system.

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A Case of an Epstein–Barr Virus (EBV) Genome-Carrying Lymphoma in an Israeli Arab Child

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Abstract—*Epstein-Barr virus (EBV) DNA (82 genome equivalents/cell) and EBNA (EBV determined nuclear antigen) were found in tumor tissue from an Israeli Arab child with Burkitt's lymphoma. A lymphoma cell line (LB-132) carrying the EBV genome was established from tumor tissue from this patient. This line resembled other previously established and characterised Burkitt's lymphoma lines. Our results suggest that EBV—carrying lymphoma which occurs endemically in Africa occurs sporadically throughout the world.*

INTRODUCTION

THE EPSTEIN–BARR virus (EBV) has been found to be associated with two human malignant diseases: African Burkitt's lymphoma (BL) and nasopharyngeal carcinoma [1–3]. About 97% of the African Burkitt's lymphoma biopsies tested contained the EBV genome, with 38 genome equivalents per cell, on the average [1–3]. It has also been shown that the African Burkitt's lymphoma represents the proliferation of an EBV-genome carrying clone, in the vast majority of the cases [4]. The relatively few non-African BL cases so far studied were EBV–DNA negative [5–7], although occasional EBV-positive cases have now been found [8, 9]. Serological evidence on a larger number of cases also failed to show the uniform anti-EBV positivity, and the approximately tenfold elevation of mean titers characteristic for the African BL. In the African BL material examined, two cases were found that lacked detectable EBV-DNA and EBNA although the patients had high serum anti EBV titers [1, 2]. The permanent lymphoid cell lines that were established from these tumors were also EBV-negative, but they could be converted to permanent EBV-DNA and EBNA positivity by *in vitro* EBV-infection [10–12]. It might be speculated that these two cases represent the same disease as the majority of the non-African Burkitt's. Since lymphomas with BL-like histology and cytology are spread throughout the world (although their clinical manifestations are slightly different from the African disease) it is conceivable that the EBV genome-negative and positive lymphomas represent etiologically different entities [5]. It may be added that only the African form shows high endemic time–space clustering. We have recently isolated and established in continuous culture a new type of lymphoid cell line from an EBV genome-negative lymphoma [13]. This line is unique because although it has B cell characteristics (surface IgM-kappa) it is devoid of EBV genome and EBV surface receptors, and thus cannot be infected *in vitro*. This line has been described in detail elsewhere [14]. It resembles, however, very closely the Su-Am-B-1 line recently isolated from an EBV genome negative lymphoma in North America [8].

The present report describes a case of an EBV-genome positive lymphoma in an Israeli Arab child, and the establishment of the lymphoma cells in continuous culture. The presence of the EBV genome was demonstrated by nucleic acid hybridization and staining by the EBV-determined nuclear antigen (EBNA).

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MATERIAL AND METHODS

Case history

A four year-old Arab boy (G.A.H.) from the Gaza strip was first seen in the Tel Hashomer clinic on February 3, 1975. During a course of one week he developed four tumours in the mandibules and maxillae, on both sides. On examination, a huge abdominal mass was palpated. No enlargement of the peripheral lymph nodes, nor liver or spleen was detected. A biopsy of the mandibular tumor revealed a classical histologic picture of Burkitt's lymphoma, of uniform cell infiltrate consisting of lymphoreticular cells, probably lymphoblasts, confirmed by cytochemical staining of imprints. Lymphoblasts were PAS negative and Sudan III positive. The blood count and bone marrow were normal except for an eosinophilia of 15%.

The patient was given cytoxan 40 mg/kg, after proper hydration and allopurinol therapy. The tumors greatly diminished in size after 48 hr and could not be seen or palpated after a week. The same dose of cytoxan was given every three weeks. Three months later (in May, 1975) an enlargement was palpated in the right testis, which suddenly grew in size two months later. On 3 August, a right orchidectomy was performed and the diagnosis of Burkitt's tumor was again confirmed.

After the operation, therapy by cytoxan was resumed. In November, 1975, the cytophosphan therapy was postponed for five weeks, and again a huge tumoral mass appeared in the right inguinal and testicular region. The administration of cytoxan, 40 mg/kg once again brought about a prompt disappearance of the tumor. It was, however, decided to add adriamycine, 60 mg/kg every three weeks for a total dose of 300 mg/m^2. The child is at present in complete remission.

Handling of the biopsies and isolation of the cell line

The biopsy material from the mandibular tumor was excised aseptically and cut into small pieces in RPMI 1640 medium containing 10% fetal calf serum and antibiotics. The cell suspension was washed several times in the medium, and partly used for the various assays (detection of EBV-DNA—by nucleic and hybridization; EBNA, Concanavalin A tests and determination of B and T cell surface markers) and in part for cell culturing. The biopsy from the tumor of the testis was handled in a similar manner.

Cultures of jaw biopsy cells were incubated in glass tubes (150 × 15 mm) in 2 ml of medium, RPMI 1640 + 30% fetal calf serum (GIBCO) at 37°C, 5% CO_2 in air and 80–95% relative humidity. The medium was changed every 4–6 days until growth was established and passages of the cell line were begun [13].

At that time, the fetal calf serum concentration was reduced to 20%.

EBV-DNA Assay

Preparation of cellular DNA and the details of the procedures used for hybridization of the DNA with precalibrated ^{32}p-labeled EBV complementary RNA (cRNA) were performed as described elsewhere [1]. The hybridizations were corrected to 10 μg DNA per filter and for ^{32}p decay. To estimate the number of EBV genome equivalents per cell the molecular weight of EBV-DNA was taken as 1×10^8 and that of the cellular DNA as 4×10^{12}. EBV DNA positive (Raji DNA) and negative (Molt-4 DNA) controls were included in all experiments. All determinations were performed at least twice.

EBNA Assay

EBNA was assayed by anticomplement immunofluorescence as described by Reedman and Klein [15]. EBNA tests were performed on the tumor biopsy cells and on the permanent cell line. Before testing, the cultures were put on a Ficoll-Hypaque gradient [16] to remove dead cells. Smears were prepared and fixed by acetone–methanol as described. The Hyland goat antiserum to human β1C/β1A reagent was used to stain EBNA at a dilution 1:40. The smears were counter stained with Evans blue (100 μg/liter) for 10 min.

Surface receptors

Rosette formation with sheep erythrocytes (E) was performed according to Jondal *et al.* [17].

Rosette formation with antibody coated sheep erythrocytes (EA) for detection of Fc receptors was performed according to Yoshida and Andersson [18].

Immunoglobulins and β2-microglobulin

Viable cells were exposed to rabbit sera with specificity for human gamma and mu heavy chains, light chains and β2-microglobulin. After washing antibody attachment was tested by incubation with FITC conjugated goat anti-rabbit immunoglobulin serum (Hyland laboratories, U.S.A.). The procedure

for surface fluorescence test was described before [19].

Assay for binding of fluorescent Con. A

Fluorescein-isothiocyanate-conjugated ConA (F-Con.A) was prepared by Miles Yeda (Rehovot, Israel). For the experiments, cells were incubated with 100 μg/ml F-Con.A for 15 min at 37°C. The cells were washed with phosphate-buffered-saline (PBS) and the fluorescence was determined on a drop of cells with a Zeiss ultraviolet microscope. Five hundred cells were counted for each point and only single cells and very small clumps (2–5 cells) were counted for the percentage of caps [13].

Assay for agglutination

Concanavalin A was obtained from Miles-Yeda. 0·5 ml Con.A at different concentrations diluted in PBS was mixed with 0·5 ml of cell suspension to give a final concentration of 2×10^6 cells/ml in a 35 mm petri dish. The density and size of aggregates was scored in a scale from − to + + + + after 30 min incubation at 24°C [13].

RESULTS

Characterization of the cells from the biopsy material

Cells obtained from the primary mandibular tumor were examined for various properties after Ficoll–Hypaque gradient centrifugation [16] to remove dead cells. Cell viability as determined by trypan blue exclusion was 95%. The cell morphology, using Wright's stain, revealed a predominance of small immature lymphocytes with large nuclei and frequent lipid droplets in the cytoplasm. The cells were tested for EBNA and found to be positive (50–60%). Examination of surface immunoglobulin showed presence of Ig-M-kappa on about 60% of the cells.

The cells were strongly agglutinated by 10 μg/ml Con.A even at low cell concentration (10^6/ml) and had low cap forming ability (10–12%) with fluorescent Con.A [13, 20].

A second tumor biopsy was obtained from the same patient six months later. This was a highly infiltrated peritesticular tumor with numerous lymphocytes. Cell preparations of this tumor were tested for EBNA and about 60% of the cells were found positive. The tumor specimen was found to contain 82 EBV genome equivalents per cell by DNA–cRNA hydridization experiments.

Establishment in culture and characterization of the cell line LB-132

After a lag phase of about 8 weeks, the mandibular biopsy cells began to divide. Subsequently the cultures grew rapidly. A cell was established, growing in loose free floating clumps. The cells were small (10–15 μ) rounded and with irregular surface. Morphologically they resembled small immature lymphoid cells, similar to other lines established by us from African EBV carrying BL [20, 21]. The cells doubling time was 24–30 hr, and the saturation density was about 2×10^6 cells/ml. The line was designated LB-132.

The LB-132 cells were EBNA positive at the earliest passage examined (4th–6th), 1–3% of the cells were also VCA positive. The cells were strongly agglutinated by Con.A even at a concentration of 1 μg/ml and at a low cell density of 10^6/ml. Cap forming ability with fluorescent Con.A was low (about 6%) and remained low in subsequent passages. Cells carried surface bound IgM and kappa, β2-microglobulin and formed EAC but no E rosettes.

Table 1 summarizes the main characteristics of the line, compared with the original biopsy cells.

DISCUSSION

While the vast majority (approximately 97%) of African BL cases represent the malignant proliferation of an EBV-genome carrying cell clone (4), the majority of lymphomas that have been diagnosed as BL outside Africa were EBV-genome negative (5–7, 22) although a few EBV positive cases were found [8, 9]. This paper reports a case of EBV-DNA and EBNA positive lymphoma in a Israeli Arab child. In view of the fact that the viral genome was found in the tumor biopsy itself, and also in the derived line, that resembled other lymphoma lines in its morphological features [21], there can be little doubt that this is a genuine EBV-carrying BL.

As argued elsewhere [22] it is unlikely that EBV-carrying lymphomas have picked-up the virus as passenger. It is relevant in this connection that EBV-negative lymphomas have been found in EBV-seropositive patients, were established as lymphoma-representative, EBV-negative lines *in vitro*, and were susceptible to EBV induced *in vitro* conversion to EBV-genome positivity [12, 23]. This demonstrates the actual absence of virus pick-up *in vivo* under otherwise favourable conditions [11, 22]. In

Table 1. *Properties of the biopsy cells and the derived cell line LB*-132

Characteristics	Biopsy	Line*
Cell morphology	Uniform, round, highly pyrominophilic, with long, multiple, basophilic nuclei. Starry sky pattern prominent.	Small immature lymphoid cells, grow readily in suspension with much clumping. No uropods, immature nuclei, prominent cytoplasmic fat droplets.
EBNA	positive	positive
EBV-DNA	positive 82 EBV-genome equivalents per cell	N.T.
VCA	N.T.	1–3%
Rosette formation E	negative	negative
Rosette formation EAC	negative	positive
Surface IgM kappa	positive	positive
Interaction with Con.A		
Cap forming ability	low (12%)	low (6%)
Agglutinability	very strong (+ + + +)	very strong (+ + + +)

*Derived from mandibular tumor.

addition, this is also unlikely in view of the high virus neutralizing antibody titers in EBV-seropositive individuals. In all likelihood, EBV-positive lymphomas originate from an EBV-carrying cell.

It is known that EBV can convert normal B-lymphocytes with a limited life span into "immortalized" lymphoid lines, capable of continuous proliferation *in vitro*. It is likely that these cells are not yet fully fledged lymphoma cells, since chromosomal [24–26] and other morphological [21] differences were regularly found between EBV-carrying lymphoid lines of non-malignant derivation and EBV-positive lymphoma lines. However, they are at least neoplastic, as suggested by the ability of the virus to induce neoplasia in non human primate hosts [27–29] and the ability of the EBV-converted normal lymphocytes to grow progressively in nude mice and in one experimental series [30] in autologous squirrel monkey hosts. In view of the excellent rejection of EBV-carrying B-blasts in infectious mononucleosis [31] immune restrictions may be expected to play a role in limiting the proliferation of EBV-converted preneoplastic cells as well [22]. Against the background of this reasoning, the development of an EBV-carrying malignant lymphoma probably involves, in addition to the original EBV-convertion at the inception, a certain degree of immunosuppression, and in addition a subsequent cytogenetic evolution of the EBV-carrying clone to fully autonomous neoplasia.

In Africa, EBV-positive Burkitt's lymphomas occurs endemically with a pronounced time-space clustering, suggesting that some environmental factor(s) play an important role in precipitating the malignant disease. Chronic holoendemic malaria has been postulated as a possible horizontally-transmitted co-factor [32]. The non-African, EBV-positive Burkitt s lymphomas show no such clustering, as far as known, and one must therefore envisage a variety of precipitating factors, probably different in the individual cases.

In all likelihood, EBV-negative and EBV-positive lymphomas represent different etiological entities [8, 14, 22]. Their similar histology, cytology and most importantly, cytogenetics suggest that their final evolution to autonomous neoplasia may be similar, although the original "transformation" event may have been caused by different viruses in the two cases.

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Leukocyte Adherence Inhibition and Immunoreactivity in Prostatic Cancer

I. Identification of Anti-Tumour Cell-Mediated Immunity and "Blocking" Factor

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Abstract—*Modification of the recently described leukocyte adherence inhibition (LAI) test was utilized for the evaluation of anti-tumour cell-mediated immunity and the identification of "blocking" factor in* 20 *patients with prostatic cancer. Evidence of cross-reactivity of the observed anti-tumour immunity with extracts prepared from tumours of the same type and the specificity of "blocking" of the reactions for autologous sensitized lymphocytes and tumour* only *have similarly been demonstrated employing the LAI test. While the observed cross-reactivity between individual tumours, within each tumour type is in keeping with observations of anti-tumour immunity in patients with other tumours, e.g., colon, breast and melanoma, observations suggestive of a specificity of "blocking" for autologous tumour* only *is perhaps somewhat unique, of which further studies will be needed to confirm.*

INTRODUCTION

ENHANCED suppression of the proliferative response of peripheral blood lymphocytes (PBL) to the non-specific mitogen phytohaemagglutinin [1–5] and inhibition of the migration of leukocytes [6] in patients with prostatic cancer have been observed in association with autologous and allogeneic serum. The suggested presence of a serum inhibitory factor, migrating in the α_2-globulin fraction, and recent observations of the concomitant reduction of the suppressive properties of this factor in association with a favorable clinical response following cryoprostatectomy [5], have prompted study of anti-tumour cell-mediated immunity and the possible interference ("blocking") of this immunity.

MATERIAL AND METHODS

Tumour extracts

Extracts of malignant prostatic tissue were prepared by solubilization of autologous and allogeneic tissue in hypertonic (3M) KCl employing a modification of the method of Meltzer [7] and Brannen *et al.* [8]. Finely minced prostatic tissue, trimmed of extraneous material, was washed in phosphate buffered saline (PBS) pH 7·2 and lyophilized. The resulting lyophilizate was pulverized with mortar and pestle, resuspended in 10 ml of 3M KCl in 0·005 M potassium phosphate buffer, pH 7·4, per gram wet weight of original tissue, and left overnight at 4°C with intermittent agitation. The KCl tissue suspension was then centrifuged at 40,000 *g* for 60 min, after which the supernate was dialyzed overnight at 4°C against deionized water. The supernate was then re-centrifuged at 40,000 *g* for 15 min to remove the gelatinous precipitate formed during dialysis and passed through a 0·45 mμ Nalgene filter into a sterile Nalgene filter flask. The filtrate was lyophilized and reconstituted to one tenth of its original volume in sterile PBS.

The protein concentration of extracts so prepared as determined by the Lowry method [9] ranged from 1·0 to 3·8 mg/ml.

Cells and serum

Peripheral blood lymphocytes were obtained from each patient by centrifugation of the leukocyte-rich plasma of heparinized blood on

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a Ficoll–Isopaque gradient. Following washing in minimum essential medium, PBL were resuspended in RPMI 1640 medium containing 100 units/ml penicillin G and 100 μg/ml streptomycine at a concentration of 2×10^7 cells/ml.

Serum was obtained concomitantly from each patient.

Leucocyte adherence inhibition test

Employing a modification of the LAI test [10] three cultures consisting of equal volumes (0·1 ml) of the patient's PBL *plus:*

(i) Normal human serum (homologous serum) of a blood type identical to that of the patient under evaluation. This was the CONTROL and contained *no tumour extract*, permitting establishment of a baseline of normal adherence. Tumour extract (as a source of antigen), employed in (ii) and (iii) described below, was replaced by the same volume of medium.
(ii) Tumour extract and homologous serum.
(iii) Tumour extract and patient's serum (autologous serum).

were incubated at 37°C for 30 min with intermittent shaking. Each culture was then introduced independently into the chambers of Standard Neubauer haemocytometers, i.e., one culture/haemocytometer, using both chambers, and incubated at 37°C for 60 min in a humid atmosphere. The total number of cells in each chamber were then counted microscopically at a magnification of 400 × in predetermined areas. In the present study, 8 squares (0·2 mm × 0·2 mm) were counted in each chamber of the haemocytometer. Cover slips were floated off, each haemocytometer gently immersed in Hanks' balanced salt solution (HBSS) at 37°C, slowly withdrawn, and again immersed and withdrawn. Each chamber of the haemocytometer then received one drop of HBSS and was covered with a clean coverslip. The number of remaining "adherent" cells were counted in the same haemocytometer squares examined previously and the mean % of adherent cells for each culture was then determined.

RESULTS

In accordance with the description of the LAI test a representative example illustrating the application of this method for the evaluation of anti-tumour immunity and the identification of serum inhibition ("blocking") of the interaction of antigen and sensitized lymphocytes in patients with prostatic cancer is presented in Table 1.

For the evaluation of each patient, a baseline of normal % adherence as a control value was initially determined by culturing the patient's (W.H.) PBL in homologous serum in the absence of tumour extract. The patient's PBL were then cultured with autologous tumour extract and homologous serum. As shown in the example, the 17% adherence obtained in such cultures was reduced when compared to the normal % adherence of 66% obtained in the absence of tumour extract, suggesting that

Table 1. *Representative example illustrating application of the leukocyte adherence inhibition test for the detection of anti-tumour cell-mediated immunity, serum "blocking" factor, antigenic cross-reactivity and specificity of serum "blocking" factor in patient with prostatic cancer*

Source of:				
Peripheral blood lymphocytes	Tumour extract	Serum	% Adherence (mean)	Explanation
W.H.	(None)	Homologous	66	Normal adherence (control)
	Autologous*	Homologous	17	Cells reactive with autologous extract indicating immunity.
	Autologous	Autologous	72	Serum "blocking"
	Allogeneic (J.T.)*	Autologous	5	Cells reactive with allogeneic extract indicating cross-reactive immunity but absence of "blocking" by serum from W.H. demonstrating specificity of serum blocking factor for autologous tumour.

*Autologous and allogeneic designate the relationship of tumour extract and serum to the source of peripheral blood lymphocytes used.

an interaction of extract (antigen) with presumably sensitized PBL had occurred. In contrast to this the addition of autologous serum resulted, as shown in Table 1, in a return of the % adherence to a level (72%) slightly exceeding that obtained in the absence of extract, thus, suggesting the absence of interaction of antigen and cells. Absence of a detectable reaction between tumour extract and autologous PBL, presumed to be sensitized on the basis of their observed interaction with extract in absence of the patient's serum, was most likely due to the presence of a serum factor preventing ("blocking") this interaction.

Application of the LAI test as employed, in evaluating the specificity of serum mediated inhibition ("blocking") of the reaction of sensitized PBL with tumour extract is further illustrated in Table 1.

As shown, when PBL's from W.H. were reacted with an allogeneic extract from a prostatic cancer patient (J.T.), of the same tumour type, and cultured in the presence of W.H.'s serum, reactivity, i.e., interaction of cells and extract, as indicated by the low % adherence compared with that in the control, i.e., 5% vs 66%, was observed. This cross-reactivity suggested that the patient's PBL were also sensitized to antigens apparently shared in common within tumours of the same type. Observation of a lower % adherence when the tumour extract was allogeneic with respect to the patient's PBL rather than autologous, 5% vs 17%, suggested that cross-reactive immunity to allogeneic tumour was greater. However, in contrast to the high % adherence observed when PBL's from W.H. were reacted with and cultured in autologous tumour extract and serum, respectively, i.e., 72%, indicating the absence of an interaction of antigen and cells and the presence of "blocking", a low % adherence, i.e., 5%, was observed when the tumour extract was allogeneic with respect to cells and serum, indicating the absence of "blocking" and the apparent specificity of the serum "blocking" factor for autologous PBL and tumour extract.

Following this representative example illustrating the application of the LAI test and interpretation of the results, the presence of anti-tumour immunity, serum "blocking" factor, antigeneic cross-reactivity of this immunity, and specificity of "blocking" factor were evaluated in 20 patients with prostatic cancer. The results of this evaluation are summarized in Table 2.

In accord with the explanation for the results illustrated in Table 1, significantly ($P < 0{\cdot}05$) reduced percentages of adherence obtained in the absence of tumour extract, i.e., in the control, were observed when PBL cultured in homologous serum were reacted with autologous and allogeneic tumour extracts, i.e., 18 and 22% vs 56% (Table 2). This reactivity suggested the presence of sensitization of the patients' PBL to autologous and allogeneic extracts and anti-tumour immunity.

When PBL from these patients were cultured in autologous serum and reacted with autologous and allogeneic tumour extracts, a significant reduction in the per cent of adherence from the control was observed with allogeneic, but not with autologous extract, i.e., 25 and 50 vs 57% (Table 2). Absence of reactivity when the tumour extract was autologous with respect to the patient's' PBL and serum and the significant difference in the reactivity of the patients' PBL with autologous extract when cultured in homologous vs that obtained in autologous serum, i.e., 18 vs 50% are consistent with the interpretation illustrated in Table 1, of the presence of inhibition ("blocking") of the interaction of extract and sensitized PBL by autologous serum and of the suggested specificity of the latter.

DISCUSSION

In the present study employment of the LAI test has provided further evidence suggestive of anti-tumour cell-mediated immunity in prostatic cancer observed previously by inhibition of leukocyte migration [11]. In addition,

Table 2. Reactivity of lymphocytes from 20 patients with prostatic cancer with autologous and allogeneic extracts of malignant prostatic tissue as evaluated by leukocyte adherence inhibition

Tumour extract	Mean ± S.D. percentage of adherence when patients lymphocytes cultured in:	
	Homologous serum	Autologous serum
(None)	56 ± 14	57 ± 23
Autologous	18 ± 12	50 ± 24
Allogeneic	22 ± 8	25 ± 20

the presence of a factor in the serum of the patients evaluated inhibiting ("blocking") the interaction of autologous antigen and sensitized PBL has been observed. Lymphocytes reactive with extract preparations of autologous tumour were also observed to react with allogeneic extracts prepared from tissue of the same tumour type indicating a cross-reactive antigenicity and immunity. In contrast to this cross-reactivity, the observed "blocking" effect of the serum appeared to be specific for interference of the interaction of sensitized PBL and autologous tumour, but not when the tumour extract was allogeneic with respect to the sensitized PBL.

It has been suggested [10] that the reactions observed in the LAI test, preliminary to some extent in view of the limited experimental [12] and clinical [10] trials evaluated thus far, may be related to the interaction of antigen with sensitized PBL as, e.g. in inhibition of macrophage or leukocyte migration is related to the liberation of "lymphokines", which in turn alter the adhering properties ("stickiness") of indicator macrophages [13]. We may envisage that PBL, including indicator macrophages, cultured in the presence of homologous serum and absence of antigen, maintain their adhesive properties and stick to the glass surface of the haemocytometer. Re-encounter of sensitized PBL with specific antigen, i.e., in cultures of cells, tumour extract and homologous serum, leads to the liberation of "lymphokines", altering their adhesive properties such that cells reacting with antigen no longer stick to the glass surface. However, when sensitized cells and specific antigen are cultured in the presence of serum possessing "blocking" factor, interaction of antigen with sensitized cells and the subsequent liberation of "lymphokines" is prevented. The cells thus, maintain their adhesive properties sticking to the glass surface as indicated by the high % adherence observed.

Contrary to the above suggested mechanism, recent studies [14] suggest that interaction of sensitized PBL with tumour antigen leads to cell surface alterations inhibiting attachment of the cells to glass.

While cross-reactivity between individual tumours, within each tumour type observed is in keeping with observations employing LAI in evaluation of patients with other tumours, e.g. colon, breast and melanoma [10], results suggesting that the specificity of "blocking" factor for autologous tumour in prostatic cancer patients is somewhat unique.

Studies are presently in progress to elucidate the immunochemical nature of the observed serum "blocking" factor, i.e., is it circulating antigen and/or antigen-antibody complexes? Alterations of its reactivity as related to tumour stage and the patients' clinical responsiveness, and evaluation of the tissue-specificity of the observed responsiveness of sensitized PBL from prostatic cancer patients are also being examined. It will be interesting to evaluate and compare the antigenic diversity of the extracts of carcinomatous prostatic tissue (previous studies of which have suggested malignant tissue to be antigenically deficient with respect to normal [15]) and the sensitivity of autologous and allogeneic PBL with their reactivity to extracts of normal and benign prostatic tissue. Pending the outcome of such studies, the identification of a prostatic tumour-specific or tumour-associated antigen is a possibility in view of the present preliminary observations.

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Presence of Splenic Suppressor Cells in Mice Bearing Regressively Growing Moloney Sarcomas and their Absence in Progressor Mice

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Abstract—*Cultured spleen cells prepared from mice bearing Moloney sarcoma at the stage of peak tumor size did not show induction of blastogenesis by Concanavalin A and phytohemagglutinin as measured by ^{3}H-thymidine uptake. Moreover, in such cultures the spontaneous ^{3}H-thymidine uptake was markedly depressed in the presence of the mitogens, especially of phytohemagglutinin. However the concomitantly assessed activity of these spleen cells in a ^{3}H-proline microcytotoxicity assay was strong. The depression of the mitogen response was restricted to the peak tumor size phase of the Moloney sarcoma and could not be observed during development and regression of the tumor. Spleen cells of mice bearing a non-regressing tumor with a development comparable with that of the Moloney sarcoma in the stage of progression did not show a depression of mitogen response and had no cytotoxic activity. Therefore the absence of detectable cytotoxic effector cells was not due to the activity of suppressor cells, and the growth rate of a tumor seems without influence on the development of suppressor cells.*

INTRODUCTION

IT HAS previously been shown [1–4] that spleen cells from mice carrying Moloney sarcoma virus (MSV-M) induced tumors have a depressed response to phytohemagglutinin (PHA) and/or Concanavalin A (Con A). Evidence has been obtained that these suppressive effects in cultures of MSV-M spleens are due to suppressor cells. The precise nature of these suppressor cells is not known. They are discussed as cells of the monocyte/macrophage series [2] and of B lymphocytes [1, 5], respectively. Gorczynski [1] found that these suppressor cells nonspecifically inhibited the PHA response of normal cells and the response of immune T cells to tumor associated antigen as measured by inhibition of migration and stimulation of protein synthesis. However, Kirchner *et al.* [4] comparing the cytotoxic activity of MSV-M spleen cells using a Chromium release assay with their mitogenic responsiveness observed coincidence between the peak of suppressor cell activity and that of specific cytotoxic effector cells, i.e., at the peak tumor phase. It was demonstrated, that the suppressor cells are *in vitro* without effect on the effector phase of specific cytotoxicity and evidence was presented suggesting that the suppressor cell activity inhibits proliferation dependent lymphocyte functions [4]. It was suggested that the suppressor cells may have the potential of interfering with the generation of cytotoxic effector cells, and it was assumed that in the MSV-M system effector cells may be generated before a significant number of suppressor cells have been developed [4]. In contrast to this assumption the present study demonstrates that in mice bearing progressively growing tumors the absence of cytoxic effector cells as measured in a ^{3}H-proline-microcytotoxicity assay is neither due to the presence of suppressor cells nor to the growth rate of the tumor. Furthermore, it is shown that unfractionated spleen cells from mice carrying regressing Moloney sarcomas in the stage of peak tumor size not only exhibited a depression of mitogen responsiveness but the mitogens even caused a decrease of spon-

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taneously occurring ^{3}H-thymidine (^{3}H-TdR) uptake; these spleen cells had a strong cytotoxicity.

MATERIAL AND METHODS

Animals and tumor cells

The STU-inbred mice and the Moloney sarcoma virus (MSV-M) producing ascitic tumor (asc-MSV-M) cells used are the same as those in preceding studies [6–8]. In addition, a progressively growing tumor was used which originated in a mouse after the regression of a MSV-M induced tumor at the site of the primary tumor and is designated "secondary tumor" (ST). This tumor obviously does not release C-type particles but contains rescuable sarcoma virus genom. As revealed in transplantation protection studies the ST cells did not induce a significant immunity against ST and asc-MSV-M cells. But in asc-MSV-M regressing mice the development of ST tumors was prevented if a small number of ST cells was used for tumor induction (to be published).

Tumor induction, spleen cell preparation, ^{3}H-proline-microcytotoxicity assay (MA)

Tumors were induced by asc-MSV-M or ST by intramuscularly (i.m.) inoculation of 10^5 and 10^6 cells, respectively, in 0·1 ml PBS into a thigh. The asc-MSV-M cell induced tumors reached their peak size during the 9th–13th day post transplantation (p.t.) and regressed thereafter. ST cell induced tumors were palpable about one week p.t. and exhibited progressive growth.

The preparation of lymphoid spleen cell suspensions as well as the ^{3}H-proline-MA have been described previously [6, 7]. The following cells were used as target cells:

(a) A cell line derived from asc-MSV-M cells; these cells produce in relatively high amounts Moloney helper virus [7].
(b) A cell line originating from ascitic ST cells; cells of this line do not produce C-type particles and grow progressively in mice after i.m. inoculation. It was demonstrated that in these cells the MSV-M genome could be rescued by infection with Moloney helper virus (to be published).

Mitogens

Phytohemagglutinin-M (PHA-M, Difco Laboratories, Detroit, Michigan, USA) was reconstituted to a 5 ml stock solution as proposed by the manufacturer. The reconstituted solution was stored in small samples at −20°C. For comparison PHA-P (Difco Laboratories, Detroit, Michigan, USA) was also used. Concanavalin A (Con A, Serva, Heidelberg, Germany) was dissolved in Eagle's minimum essential medium (MEM) to prepare solutions containing 100 μg/100 μl and was stored in small samples at −20°C until use and further dilution. Preliminary titration studies in spleen cell cultures of normal STU-mice revealed that Con A concentrations of 2–5 μg/culture and PHA-M dilutions of the stock solution between 1:2·5–1:10 were optimal with regard to a mitogenic response.

Determination of mitogen induced proliferative response in vitro

For the investigation of the mitogenic response, lymphoid spleen cells were cultured in 10 × 75 mm round bottom glass tubes at 2×10^6 nuclear cells (cells of two spleens were pooled) in 0·5 ml of MEM supplemented with 10% heat inactivated calf serum (one serum pool was used for the whole study), 100 i.u./ml penicillin and 50 μg/ml streptomycin. Mitogens or culture medium as control were added in a volume of 0·1 ml to each culture. Triplicate or quadruplicate cultures were incubated at 37°C in a stationary upright position after gassing with 5% CO_2 in air and closed with silicone rubbers. After 26–28 hr, usually 1 μCi ^{3}H-thymidine (^{3}H-TdR, 6-^{3}H-thymidine; specific activity 22·3 Ci/m mole; Radiochemical Centre, Amersham, England) was added to each tube and cultures were incubated for an additional 20 hr. The cultures were harvested onto glass fiber filters (2·5 cm GF/C; Whatman, London, England), washed three times with PBS and precipitated with 10% TCA and 5% TCA. The glass fiber filters with precipitates were placed in vials and treated by soluene 350 (Packard, Frankfurt, Germany), and assayed by liquid scintillation spectrophotometry (TRICARB model 3375, Packard, Frankfurt, Germany). The arithmetical mean of each set of three respectively four cultures was determined and expressed as counts/min.

RESULTS

Mitogen response of spleen cells derived from STU-mice during different phases of asc-MSV-M induced tumor development

The responsiveness to PHA-M and Con A of lymphoid spleen cells from STU-mice at the beginning of asc-MSV-M tumor development

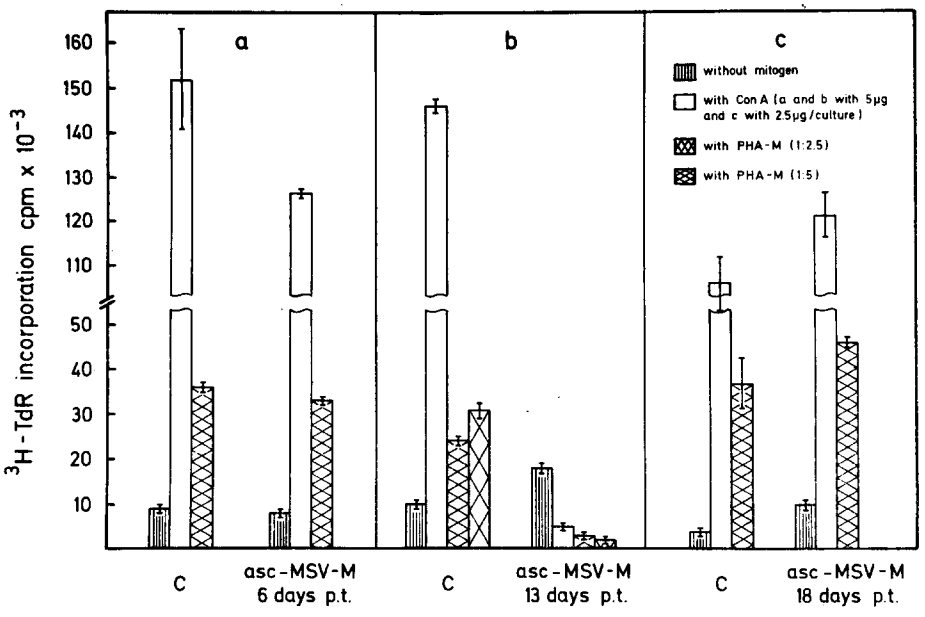

Fig. 1. Comparison of mitogen response during various stages of tumor development. Spleen cells were taken 6 days (tumor already measurable), 13 days (peak tumor phase) and 18 days (tumor in regression) after transplantation (p.t.) of ascitic MSV-M tumor cells. C: spleen cell cultures of age matched control mice. Values are mean ± standard error.

(6 days p.t.), the peak phase (13 days p.t.) and the regression period (18 days p.t.) was compared with the responsiveness of lymphoid spleen cells of age matched control mice (Fig. 1). Six and 18 days after tumor cell transplantation the mitogen response as measured by the incorporation of ^{3}H-TdR was nearly the same in spleen cells from both tumor and control mice. However, spleen cells of the peak tumor phase had a new pattern of reactivity: they did not respond to Con A or PHA-M with stimulation but with a reduction of the spontaneous ^{3}H-TdR uptake in the presence of the mitogens. When the PHA-P was used, the stimulation of spleen cells from control mice was not as high as in the case of PHA-M but there was also a depression of baseline proliferation in spleen cell preparations obtained at the peak tumor phase. The marked effect of PHA-M was the reason to use PHA-M throughout the following experiments.

Treatment of spleen cells from normal mice and mice bearing asc-MSV-M tumors at the stage of peak size with varying concentrations of PHA-M

Because spleen cells prepared from mice at the stage of peak tumor size did not show stimulation of ^{3}H-TdR uptake in the presence of mitogens in concentrations that caused a considerable stimulation in control spleen cells, an analysis was undertaken to determine whether this effect also occurs with varying amounts of PHA-M. Figure 2 shows the results.

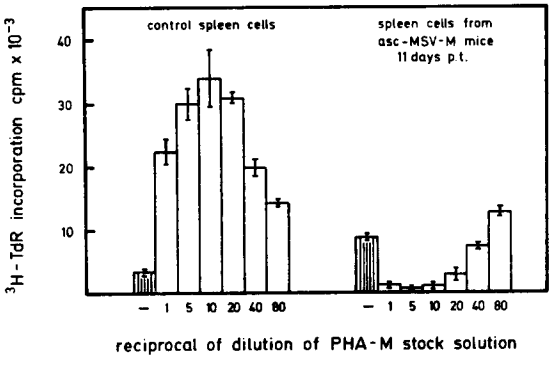

Fig. 2. Pattern of response after adding varying concentrations of PHA-M to spleen cell cultures of control mice and mice with Moloney sarcoma in its peak phase. Values are mean ± standard error.

It can be seen that PHA-M concentrations that induce optimal stimulation of ^{3}H-TdR uptake by spleen cells from control mice reduce the spontaneous ^{3}H-TdR uptake by spleen cells from peak tumor mice. Only the lowest tested PHA-M concentration (1:80 dilution of the stock solution) that caused a weak stimulation in control cultures induced a slight increase of the spontaneously occurring ^{3}H-TdR uptake in cultures of spleen cells from asc-MSV-M peak tumor mice.

Replacement of spleen cell culture fluid before addition of ^{3}H-TdR

To exclude factors that might compete with the uptake of ^{3}H-TdR and therefore could

mask a DNA-synthesis in the cultures of spleen cells from peak tumor mice, the medium was replaced before addition of ^{3}H-TdR. The spleen cell cultures were incubated for 42 hr in the presence of PHA-M, then the culture fluid was replaced by a fresh medium and 2 μCi ^{3}H-TdR was added, and the cultures were again incubated, however only for 6 hr in order to prevent a possible production of new competing factors. Under these conditions, again no mitogenic stimulation but a repression of spontaneous uptake of ^{3}H-TdR was detected in cultures of spleen cells from asc-MSV-M peak tumor mice (Fig. 3). The replacement of the culture medium caused in control cultures with or without mitogen as well as in cultures

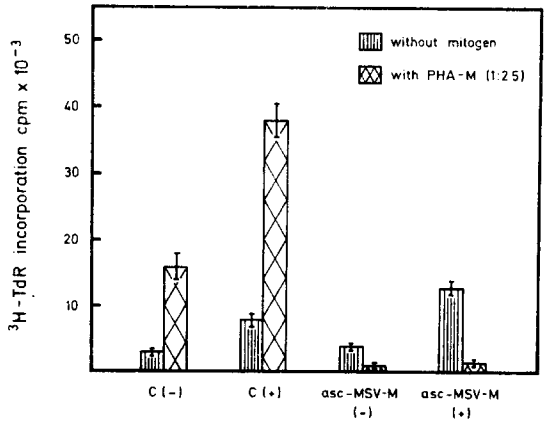

Fig. 3. Replacement of culture fluid before addition of ^{3}H-TdR. Influence on the isotope uptake of spleen cell cultures of asc-MSV-M peak tumor mice (11 days p.t.) and control mice (C) cultivated in the presence or absence of PHA-M for 42 hours. Values are mean ± standard error. (—): without change of culture medium before addition of ^{3}H-TdR (2 μCi/culture for six hours); (+): addition of ^{3}H-TdR after replacement of culture fluid by fresh medium.

of spleen cells from peak tumor mice cultured without PHA-M an increased uptake of ^{3}H-TdR.

Mitogen response of mixtures of spleen cells from peak tumor mice and syngeneic control mice

It was then of interest to determine whether spleen cells from peak tumor mice could suppress the Con A and PHA-M response of spleen cells from normal mice. Thus, 1×10^6 spleen cells from peak tumor mice in 0·25 ml culture medium and 1×10^6 normal spleen cells in 0·25 ml medium were mixed and the response to the two mitogens was compared with the response of 2×10^6 normal spleen cells in 0·5 ml and 2×10^6 peak tumor spleen cells in 0·5 ml medium. In the mixed cultures the spontaneous ^{3}H-TdR uptake (Fig. 4) was nearly as high as

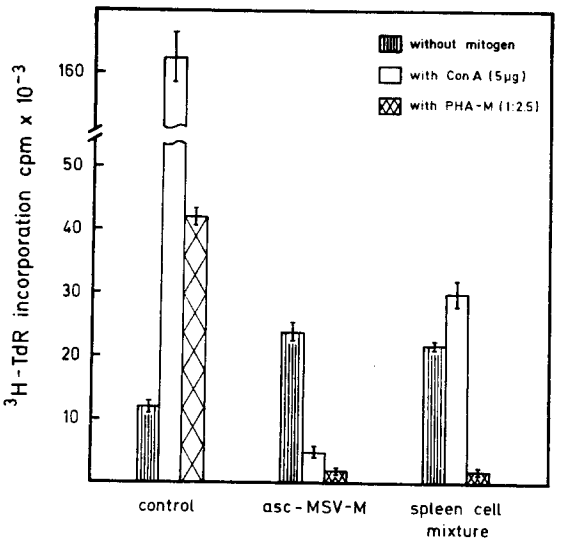

Fig. 4. Effect of mixing spleen cell suspensions of asc-MSV-M peak tumor mice (11 days p.t.) and of syngeneic control mice (C) on the mitogen response. The cell mixture consisted of equal parts of each suspension. Values are mean ± standard error.

in the cultures of the peak tumor spleen cells alone, and the PHA-M induced suppression of the uptake of ^{3}H-TdR was comparably strong in cultures of both groups. Only Con A induced a small increase of ^{3}H-TdR uptake in the mixed cultures but with a rate far below to that determined in the control cultures.

Comparison of mitogen response and cytotoxic activity of spleen cells from mice bearing asc-MSV-M cell and ST cell induced tumors in comparable stages

To see whether spleen cells from mice with a progressively growing tumor (ST) also exhibit a depressed uptake of ^{3}H-TdR after mitogen stimulation, the following experiment was conducted: Spleen cells from STU-mice bearing ST tumors with a diameter of about 1·3 cm were tested 14 days p.t. and compared with spleen cells of asc-MSV-M mice 13 days p.t. that beared tumors with diameters of about 1·15 cm. In contrast to the spleen cell cultures from mice with asc-MSV-M cell induced tumors the mitogen response of spleen cells derived from mice with ST tumors was similar to that of control cultures (Fig. 5, lower part). The concomitantly assessed cytotoxic activity in the ^{3}H-proline-MA [7] using the asc-MSV-M-derived cell line revealed a strong reduction of the number of attached target cells by spleen cells from asc-MSV-M bearers whereas such an effect was not observed after exposing these target cells to spleen cells from ST bearing mice (Fig. 5, upper part). Also there was no cytotoxic activity of spleen cells from mice

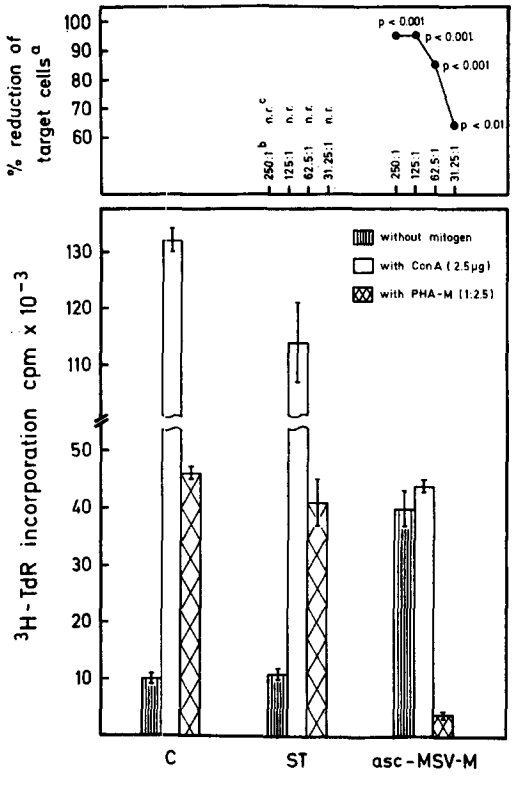

Fig. 5. Comparison of the mitogen response and the cytotoxic activity in the ^{3}H-proline microcytotoxicity assay of spleen cells from mice either carrying ST (14 days after transplantation, tumor diameter 1·3 cm) or asc-MSV-M (13 days after transplantation, tumor diameter 1·15 cm) tumors. Asc-MSV-M-derived cells were used in this experiment as target cells.

(a) % Reduction of target cells was measured by determination of the ^{3}H-proline radioactivity taking as 100% the mean radioactivity of control cultures which had received identical numbers of spleen cells from normal mice (C).

(b) Ratio of nuclear spleen cells to target cells.

(c) No reduction of target cell radioactivity in comparison to normal spleen cells.

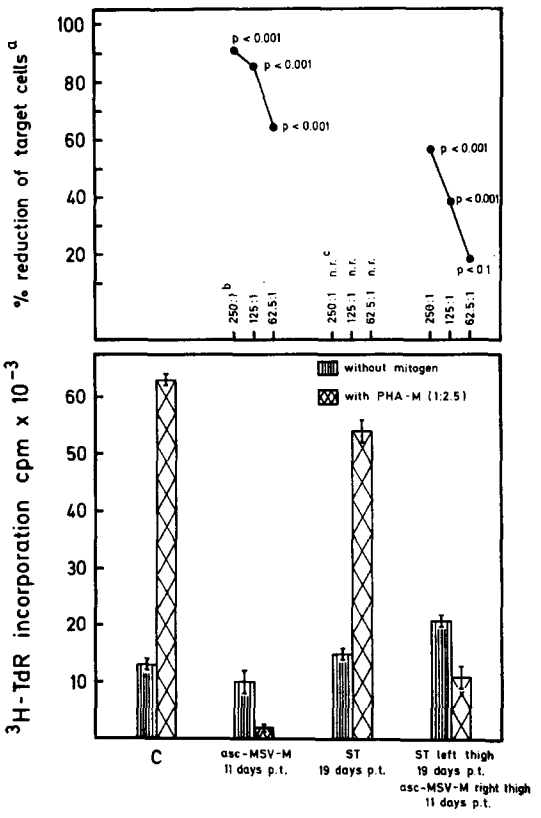

Fig. 6. Cytotoxic activity and loss of mitogen response of spleen cells from mice in which both ST tumor and 8 days later asc-MSV-M tumor have been induced (a, b and c), see legend to Fig. 5.

bearing ST tumors when a cell line derived from ascitic ST cells was used as target cells.

After these results the question arose whether the ST tumor prevents the appearance of spleen cells with cytotoxic activity and depressed mitogen response after inoculation with asc-MSV-M cells. Mice were inoculated with ST cells in the left thigh and received asc-MSV-M cells eight days later in the right thigh. Mice without inoculations and mice receiving either ST cells or asc-MSV-M cells alone served as controls. Eleven days p.t. of asc-MSV-M cells the tumors had developed at the site of inoculation (Fig. 6); at this time spleen cells were taken from mice of the four groups and assayed for PHA-M response and cytoxic activity in the ^{3}H-proline-MA. As Fig. 6 shows, asc-MSV-M tumor development induced in ST tumor bearers and non-bearers both loss of mitogen responsiveness of spleen cell cultures and spleen cells with cytotoxic activity in the ^{3}H-proline-MA.

DISCUSSION

The present studies confirm the observations of Kirchner *et al.* [3] that spleen cell cultures of mice bearing primary MSV-M-induced tumors show a marked depression of PHA and Con A reactivity. As in the investigation of Kirchner *et al.* [2, 3], we also found a parallelism between the depression of the mitogen response and the development of the tumor, and a coincidence between the peak of suppression of mitogen reactivity and that of cytotoxic effector cells. The frequently used assessment of lymphocyte stimulation by mitogen(s) as an indication of cellular immune potential is not in agreement with these results, at least with regard to the peak tumor phase. Moreover, instead of reflecting a state of immunological deficiency the PHA unresponsiveness seems to reflect evidence of an intense immune response. The spleen cell preparations of MSV-M-induced tumor bearers were able to inhibit mitogen response of normal spleen cells; this observation is in accordance with

that of Gorczynski [1] and Kirchner *et al.* [2] and indicates a lack of immunologic specificity of the mitogen suppression phenomenon.

In contrast to the findings of Kirchner *et al.* [2, 3] we observed not only a depression of mitogen reactivity in the MSV-M spleen cell cultures but the mitogens even reduced the "spontaneous" (mitogen-independent) ^{3}H-TdR uptake in such cultures. This discrepancy may be due to the different inbred mice strains used.

We found that relatively high doses of PHA inducing maximal stimulation of normal spleen cell cultures caused in MSV-M spleen cell cultures a decrease of the spontaneous ^{3}H-TdR uptake whereas low concentrations induced a slight mitogen response. These results indicate that a mitogen consuming process is not operative in the phenomenon of unresponsiveness of MSV-M spleen cell cultures to PHA.

To exclude factors in the supernatant of spleen cell cultures that may compete with ^{3}H-TdR and cause failure of ^{3}H-TdR uptake in spite of the presence of DNA stimulation [9], the influence of replacing the culture medium before addition of ^{3}H-TdR was investigated. This procedure did not increase the ^{3}H-TdR uptake in spleen cell cultures of MSV-M peak tumor mice cultivated in the presence of PHA. However, spleen cell cultures of MSV-M peak tumor mice cultivated in the absence of PHA and that of normal mice cultivated with or without PHA showed an increased ^{3}H-TdR uptake.

Kirchner *et al.* [2] reported that spleen cells of both MSV-M progressors and regressors exhibited specific cytotoxicity and a suppressed PHA response. In spleens from mice carrying a Rauscher leukemia virus-induced ascitic tumor, the PHA response was also strongly suppressed, but there was only low cytotoxic activity [2]. Furthermore, in spleen cells from rats injected with cells from the Gross leukemia virus induced lymphoma (C 58 NT) D suppression of mitogen response was only found if the cells were taken from progressors; these cells were unresponsive in the mixed leukocyte tumor cell interaction (MLTI), but became responsive in the MLTI and to mitogen after passage over rayon adherence columns or pretreatment with an iron/magnet technique [10]. Based on these results it was assumed [4] that the suppressor cells may interfere with the immune response against some tumors by inhibiting the proliferation of effector cells. The demonstration of suppressor cells in MSV-M regressor mice does not fit in this picture, and for an explanation it was pointed out [10] that in the MSV-M system tumors reach a large size before regression starts, while regressing (C 58 NT) D tumors are quite small in relation to the body weight of the rats. This explanation is based on the assumption that activation of suppressor cells may be mediated by substances released from the tumor, which only reach sufficient levels after the tumor has attained a certain size.

However, neither the assumption of an interfering activity of suppressor cells on the generation of effector cells nor the influence of tumor growth rate and size on the development of suppressor cells were confirmed in our experiments. We found no cytotoxic effector cell activity in the spleen cell cultures from mice with a non-regressing tumor (ST) in spite of the absence of suppressor cells, and the development of this ST tumor in size was comparable with that of MSV-M induced tumors during the stage of progression which was accompanied by the appearance of suppressor cells.

This ST tumor developed at the site of a primarily regressed MSV-M induced tumor possibly after immune selection of nonproducer tumor cells that may originate besides C-type particles producing tumor cells after infection with murine sarcoma virus [11]. Nonproducer tumor cells may exhibit no demonstrable immunity [12] or only weak antigenicity after repeated immunizations [13]. The failure of ST tumor cells to induce a demonstrable immune reaction may be also the cause of the failure to induce suppressor cells. Spleen cell cultures from mice bearing at one site a MSV-M regressing tumor and at the other site a progressing tumor showed a suppressed mitogen response. This indicates that the progressively growing tumor did neither prevent the phenomenon of depressed mitogen response and of reduction of the spontaneous ^{3}H-TdR uptake in the presence of mitogens nor the induction of cytotoxic effector cells. The function of the suppressor cells *in vivo* and their precise nature remains to be determined.

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Effects of 5-Tungsto-2-Antimoniate in Oncogenic DNA and RNA Virus-Cell Systems*

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Abstract—*We examined the effects of the heteropolyanion* 5-*tungsto*-2-*antimoniate on* Herpes virus saimiri, *Epstein–Barr virus, purified murine and avian viral reverse transcriptases, and on highly purified DNA polymerases α and β from lymphoblastoid cells producing Epstein–Barr virus or* Herpes virus saimiri. 5-*tungsto*-2-*antimoniate enhanced the replication and cytopathic effect of* Herpes virus saimiri *when the virus was present prior to treatment with the compound. Moreover, a rhesus cell line chronically infected with* Herpes virus saimiri *showed enhancement of cytopathic effect, early and late viral antigens and virus production. In contrast, the compound had no effect on EBV replication or antigen production in lymphoblastoid cell lines, whether the EBV was transforming or nontransforming. Highly purified Rauscher leukemia virus and avian myeloblastosis virus reverse transcriptases were inhibited to different degrees with a greater effect on the mammalian enzyme. The great susceptibility of the murine reverse transcriptase may explain the inhibition of* de novo *infection, with murine oncornavirus, of cells in medium containing the compound. Highly purified DNA polymerases α and β from P3HR–1 and MLC–1 cells also showed differential inhibition with a greater effect on the β enzymes. The different effects of this compound on oncogenic DNA and RNA virus systems suggest a complex mechanism of action and require further study. In the case of HVS, TA may interfere with the synthesis or action of a cellular product inhibitory for virus replication.*

INTRODUCTION

THE HETEROPOLYANION 5-tungsto-2-antimoniate (TA) is an antiviral agent which inhibits murine leukemia and sarcoma viruses, both *in vitro* (Sinoussi *et al.*, in preparation) and *in vivo* [1]. It protected mice against Friend- and plasma variant-induced leukemias, and delayed the appearance of tumors in newborn mice inoculated with Moloney murine sarcoma virus [1]. TA is an inhibitor of murine reverse transcriptase [2]. In addition, TA acts against many DNA and RNA nononcogenic viruses both *in vitro* and *in vivo* [3,4].

In the present experiments, we studied the effects of TA on two oncogenic agents, *Herpes virus saimiri* (HVS) [5,6] and Epstein–Barr virus (EBV) [7]. We also studied the effects of TA on highly purified Rauscher leukemia virus (RLV) and avian myeloblastosis virus (AMV) reverse transcriptases, and on cellular DNA-dependent polymerases α and β from primate cell systems.

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MATERIAL AND METHODS

5-*Tungsto*-2-*antimoniate*

The ammonium salt of this compound was prepared according to the method described in [1] and [8]. We used TA dissolved in buffered saline, as this did not differ in biologic effects, in experiments with HVS and EBV, from TA dissolved in water. TA was dissolved in water for DNA polymerase inhibition studies.

Cell cultures

We used continuous owl monkey (*Aotus trivirgatus*) kidney cells kindly supplied by Dr. L. V. Meléndez; MEST–HVS, spontaneously transformed rhesus monkey (*Macaca mulatta*) embryo cells chronically infected with HVS [9,10]; and Vero, a continuous line of African green (*Cercopithecus aethiops*) monkey kidney cells. We also used Raji [11], an EBV-nonproducer lymphoblastoid cell line which contains the EBV genome; HK–LY 28 [12] and P3HR–1, EBV lymphoblastoid cell lines which produce transforming and nontransforming virus, respectively; and MLC–1, a lymphoblastoid cell line derived from a lymph node of a marmoset monkey (*Saguinus oedipus*), infected with HVS [13,10]. About 47 genome equivalents of HVS are present in each MLC–1 cell and 12–15 genome equivalents have also been detected in MEST–HVS cell cultures. (Ablashi, D. V., Fleckenstein, B. and zur Hausen, H., unpublished observations.)

Adherent cells were maintained in Eagle's Minimal Essential Medium (Grand Island Biological Co., Grand Island, New York) containing 100 units per ml of penicillin, 100 μg/ml streptomycin, and 2% heat-treated (56°C for 0·5 hr) fetal calf serum. Lymphoblastoid cells were maintained in medium RPMI–1640 containing penicillin, streptomycin and 20% heat-treated fetal calf serum. All cells were incubated at 37°C.

HVS

HVS, originally supplied by Dr. L. V. Meléndez, was plaque-purified three times and repeatedly passaged in our laboratory. A virus pool, having a titer of $10^{5\cdot5-6\cdot0}$ 50% tissue culture infective doses ($TCID_{50}$) per ml, was prepared in owl monkey kidney (OMK) cells and used in these experiments. One ml of this virus pool induced malignant lymphoma and lymphocytic leukemia in an adult owl monkey. HVS early antigen (EA) and late antigen (LA) were assayed by standard indirect immunofluorescent techniques [14].

EBV

EBV preparations were titrated by induction of EBV early antigen in Raji cells [15] or by induction of nuclear antigen in lymphocytes from human umbilical cords [16]. These antigens, as well as viral capsid antigen [17] were assayed by standard immunofluorescent techniques.

Determination of drug toxicity and effect on virus

Confluent tube cultures of OMK or Vero cells were employed. In toxicity studies, four cultures were incubated with a given concentration of compound, and observed for 14 days. Assays of the effect of TA on HVS replication were performed with cell cultures inoculated with 0·2 ml of ten-fold dilutions of the virus pool. Compound was added to all cultures, under the conditions specified for each experiment. Four cultures were used for each dilution of virus, and $TCID_{50}$ were calculated by the method of Reed and Muench [18]. The final reading for HVS cytopathic effect (CPE) was made 14 days after virus inoculation. Experiments with lymphoblastoid cells were performed in 75 cm^2 plastic Falcon flasks containing 35 ml of medium.

DNA polymerase assays

Assays for DNA-dependent and RNA-dependent DNA polymerases were as previously described [19, 20]. The procedures for purification of these enzymes have been described [21–24].

RESULTS

Toxicity of TA

TA had a borderline toxicity at a concentration of 40 μg/ml for Vero and OMK cells; greater concentrations caused cell rounding, refractility and death. We therefore used TA in concentrations of 10 and 20 μg/ml. For Raji, P3HR–1 and HK–LY 28 cells, borderline toxicity occurred at 25 μg/ml and cell degeneration and death occurred at greater concentrations.

Lack of effect of TA on HVS itself or on its absorption

In these experiments virus dilutions were made in medium containing 10 or 20 μg/ml TA, and were kept at 4°C for 24 or 48 hr. Aliquots of 0·2 ml were then added to Vero or OMK cell cultures for virus absorption for 2 hr. The cells were then washed three times, and fresh medium, not containing TA, was added. Cell cultures were observed for up to 14 days, with medium changes twice weekly. These experiments were repeated three times, and all experimental and control titrations yielded values of $10^{6\cdot0}$ $TCID_{50}$/ml. These results suggest that TA had no effect on HVS itself, or on its absorption or penetration.

Enhancement of HVS infectivity in OMK and Vero cells by TA

Table 1 shows that titration of HVS in the presence of TA yields titers which are thirty-fold higher than in the absence of TA. Pretreatment of the cells for 48 hr with TA, followed by titration of HVS in the presence of TA, gave similar results (Table 2). Pretreatment with TA of the cells used in the titrations thus seemed to have no additional effect (Table 2). HVS from the end-points of the TA titrations could be used to infect other cell cultures productively.

Table 1. *Enhancement of HVS replication by treatment of cells with TA after HVS absorption*

TA concentration (μg/ml)	HVS titer (log $TCID_{50}$/ml)
0	5·5
10	7·0
20	7·0

In these experiments, either Vero or OMK cell cultures were used for HVS titrations. TA was added to the cell cultures immediately after the 2-hr period for the absorption of HVS and kept on the cells for the duration of the titration. All experiments were repeated three times. Cells incubated solely with TA showed no CPE or toxicity. Cell cultures were observed for up to 14 days, with medium changes twice weekly.

Table 2. *Enhancement of HVS replication by pre-treatment and post-treatment of cells with TA*

TA concentration (μg/ml)	HVS titer (log $TCID_{50}$/ml)
0	5·5
10	7·0
20	7·0

In these experiments, cell cultures were incubated with TA for 48 hr at 37°C, washed three times, and used for HVS titrations. TA was added again to cell cultures immediately after the 2-hr period for the absorption of HVS and kept on the cells for the duration of the titration. Cell cultures were observed for up to 14 days for HVS CPE, with medium changes twice weekly. These experiments were repeated three times, using either Vero or OMK cells. Cells incubated solely with TA showed no CPE or toxicity. The results do not differ from those presented in Table 1.

Enhancement of HVS replication in MEST–HVS cells by TA

Table 3 shows that TA had an enhancing effect on HVS replication in a cell line (MEST–HVS) chronically infected with HVS. There was an increase in the percentage of cells showing CPE, an increase in the percentage of cells showing HVS antigens by immunofluorescence, and a thirty-fold increase in the amount of infectious HVS produced. The equilibrium between cellular resistance and viral replication was thus shifted in favor of the virus.

Table 3. *Enhancement of HVS replication and antigen production (HVS–EA and LA) by TA in a chronically infected cell line (MEST–HVS)*

TA concentration (μg/ml)	Cytopathic effect	HVS antigen production (% of cells)	Titer of HVS (log $TCID_{50}$/ml)
0	1+	5–7	0·5
10	3+	25–30	2·0
20	3+	25–30	2·0

Cells were maintained for 5 days at 37°C in the presence of TA. 1+ = 5–25% of cell sheet involved; 3+ = 50–75% of cell sheet involved. The HVS antigens (LA and HVS–EA) were assayed by immunofluorescence. LA was predominant. For the determination of virus titer, cells were removed from the surface of the containing flask, rapidly frozen and thawed three times with their supernates and titrated in owl monkey kidney cells. Cell cultures were observed for up to 14 days for HVS CPE, with medium changes twice weekly. These experiments were repeated twice.

Lack of effect of TA on EBV replication

Treatment of Raji, P3HR–1, or HK–LY 28 cells with TA failed to induce changes in the production of EBV or its antigens. However, treatment of P3HR–1 cells caused an increase in the size of the giant cells which occur in P3HR–1 cultures (Figs. 1–4).

Inhibition of DNA polymerases by TA

Figure 5 shows that RLV RNA-dependent DNA polymerase was inhibited 50% at a concentration of 2 μg TA per ml of reaction mixture, whereas the AMV enzyme required 100 μg TA per ml for 50% inhibition. At concentrations of 10 μg TA per ml and above, the RLV enzyme was completely inhibited. Evidence for the reversibility of the inhibition of the murine reverse transcriptase has been found by J.-C. Chermann *et al.* [2]. The nuclear (β) and cytoplasmic (α) DNA dependent DNA polymerases from P3HR–1 cells were also differentially inhibited. Fifty percent inhibition was seen at 2·5 and 5·0 μg TA per ml of reaction mixture, respectively. Thus, cellular as well as viral enzymes were affected by this compound. However, in contrast to its effect on the RLV enzyme, TA did not completely

inhibit the cellular enzymes, even at relatively high concentrations of the compound.

We also found that the nuclear and cytoplasmic DNA dependent DNA polymerases of MLC–1 cells were differentially inhibited. This inhibition was almost identical to that for the polymerases derived from P3HR–1 cells.

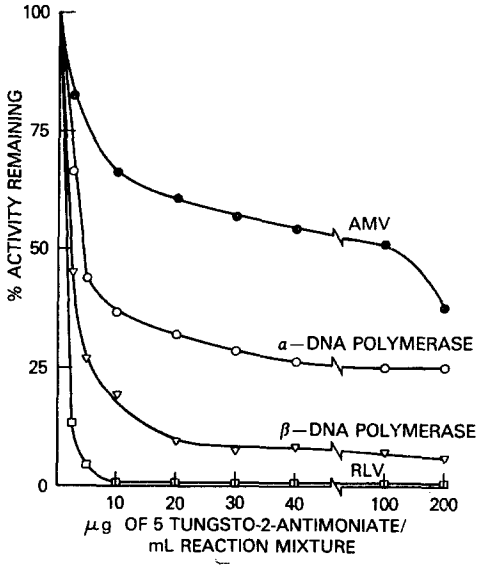


Fig. 5. Reaction mixtures (100 μl) containing the indicated amounts of TA were initiated by the addition of enzyme and incubated for 30 min at 37° and the incorporation of ^{3}[H]thymidine monophosphate (TMP) into acid insoluble material was determined. Specific activities for the enzymes were 65 pmole TMP/ng/hr for avian myeloblastosis virus reverse transcriptase, 40 pmole TMP/ng/hr for Rauscher leukemia virus reverse transcriptase, and 16 pmole TMP/ng/hr for the α DNA polymerase of P3HR–1 cells. Similar results were seen for DNA-dependent DNA polymerases β and α from P3HR–1 cells using either 20 μg activated DNA or 5 μg d(AT) per ml of reaction mixture as template. RNA-dependent DNA polymerase from AMV and RLV were assayed using $rA{\cdot}dT_{10-12}$ (2·5 μg/reaction) as template. A similar inhibition was also seen using rC·dG as template.

DISCUSSION

The results of the HVS titrations in the presence and absence of TA indicate that potentially infectious doses of HVS are present beyond the normal end-point of an HVS titration. It was also found that TA had no demonstrable effect on HVS itself, or on its absorption or penetration. These results could possibly be explained by the presence of an inhibitor, the action of which is overcome by relatively large doses of HVS, and which is sensitive to the presence of TA.

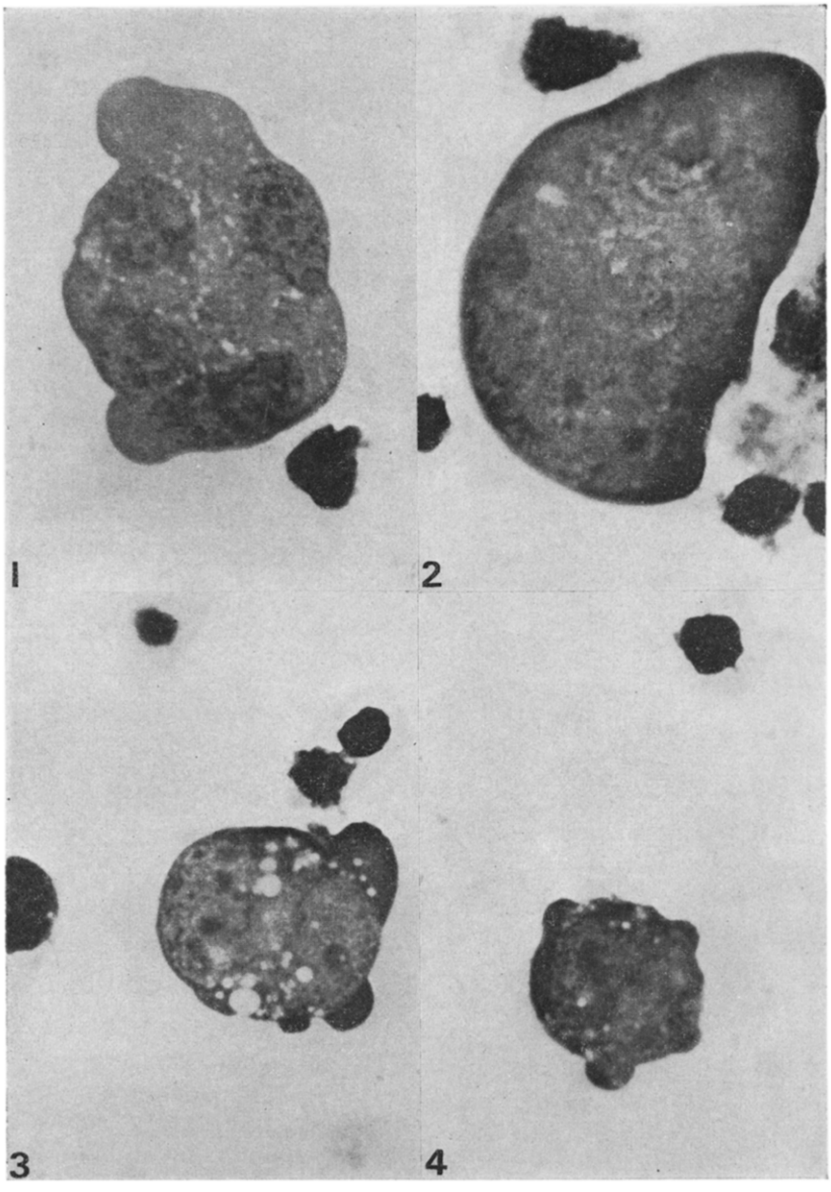
It had been previously found [25] that pretreatment of human embryonic lung cells with IUdR enhanced the replication of human cytomegalovirus. Virus genome in drug-treated cells exhibited a shorter eclipse period and the cells produced more infectious virus sooner than did untreated cells. In experiments in which the multiplicity of infection was varied, it was found that there was a hundred-fold increase in the sensitivity of cultures for virus detection when the cells had been previously exposed to IUdR. The authors felt that IUdR interfered with the synthesis of a cellular product inhibitory for cytomegalovirus replication, and that this product probably was not interferon. Such inhibitors have been described [26]. We feel that TA may have an effect on HVS replication similar to that of IUdR on the replication of cytomegalovirus.

Additional evidence for the presence of an inhibitor was provided in another communication [10], in which it was found that MEST–HVS cells are super-infectible with HVS and yielded thirty-fold more infectious virus than control cells. This suggests that a viral inhibitor is present in MEST–HVS cells, and that the action of this inhibitor is overcome by a relatively large dose of HVS.

In contrast to its effect on HVS, TA had no effect on the replication of, or antigen production by, either transforming EBV (HK–LY 28 cells) or nontransforming EBV (P3HR–1 cells). We have no explanation for the different results in the EBV and HVS cell systems in view of the fact that HVS otherwise is an excellent model for EBV. We also have no explanation for the larger size of the giant cells in the P3HR–1 cultures treated with TA. We do not know if the larger giant cells formed as a result of cell agglutination; dextran sulfate has caused trypsin-dispersed HeLa cells to form clumps [27]. In contrast to the results with TA, phosphonoacetic acid, another viral inhibitor, has been found to inhibit the replication of both EBV and HVS [28].

The inhibition by TA of the RNA-dependent DNA polymerase of RLV confirms the work of Chermann *et al.* [2], who also found that TA inhibited *E. coli* DNA polymerase and RNA polymerase. Reverse transcriptase is needed for oncornavirus replication during the primary infection of cells. The great susceptibility of the murine reverse transcriptase to TA may explain the important inhibition of *de novo* infection, with murine oncornavirus, of cells in a medium containing TA at relatively low concentrations (Sinoussi *et al.*, in preparation). The marked inhibition of the murine reverse transcriptase may thus be related to the profound *in vitro* and *in vivo* inhibition of certain murine oncornaviruses.

The finding that TA partially inhibits the α



Figs. 1 *and* 2. *P3HR–1 cells treated with* 10 *μg/ml TA. The giant cells have one to several nuclei. Giemsa,* × 800.

Figs. 3 *and* 4. *Control P3HR–1 cells. The cells are similar to those shown in Figs.*1 *and* 2, *only smaller. Giemsa,* × 800.

and β DNA polymerases of primate cells provides evidence that this compound, in what seems to be nontoxic concentrations, may interfere with the nucleic acid metabolism of these cells. Other investigators [29] found that treatment with IUdR greatly reduced cellular DNA synthesis, and felt that this effect may have played an important role in the facilitation of viral replication in cells pretreated with this compound. Perhaps a similar effect, which may lead to interference with the production or action of viral inhibitors, is operative in the case of TA and HVS.

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Cross-reaction between Antigens of Human Myelogenous Leukemia and Mason–Pfizer Monkey Virus*

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Abstract—*An antigen similar to a major protein of Mason–Pfizer monkey virus (MPMV) was found in breast cancers of 2 out of 9 women and in splenic tissue or peripheral leukocytes from 12 of 12 patients with myelogenous leukemias. Such antigen was not detected in other tumors or normal tissues. The cross-reactivity was detected using inhibition of the indirect quantitative radioimmunoprecipitation of ^{125}I-labeled purified MPMV p25 and anti-MPMV antiserum. Particles banding in a density region of 1·15 to 1·17 g/ml, the density characteristic of the known animal leukemia viruses, were used as inhibitors for the precipitation. An antigen isolated from a spleen of a patient with myelogenous leukemia using anti-p25 immunoabsorbent column was similar in net electrical charge and molecular weight to MPMV p25. These results suggest that a virus-like particle bearing some antigenic relatedness to MPMV may be potentially important in the pathogenesis of human myelogenous leukemia.*

INTRODUCTION

THE MASON–PFIZER monkey virus (MPMV), isolated from a spontaneous mammary carcinoma occurring in a Rhesus monkey [1], possesses morphological, biochemical and biophysical characteristics of known RNA tumor viruses [2–4]. The virus has been successfully propagated by cocultivation of the original mammary tumor with monkey embryo cells and by infection of chimpanzee and human cells [5,6]. MPMV-like particles have been identified in human cell lines derived from patients with leukemia or other malignancies [7, 8, 9] and in lines derived from normal cells which have spontaneously transformed [10, 11]. I report here that an antigen can be isolated from spleen cells of patients with myelogenous leukemia (ML). The antigen cross-reacts with and has very similar net electrical charge and molecular weight to MPMV p25. The results are consistent with the possibility that a MPMV-like virus may be involved in the etiology and/or pathogenesis of human myelogenous leukemia, a possibility strengthened by the demonstration of antibody to MPMV p25 in plasma from patients with ML [12].

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MATERIAL AND METHODS

Viruses

The MPMV was propagated in either normal human lymphoblastoid cell line NC37 or the original monkey mammary tumor cell line, MT. A preparation purified by sucrose density gradient centrifugations was obtained from the J. L. Smith Memorial for Cancer Research, Pfizer, Inc. The virus was further purified by centrifugation through a 5-ml column of 30% sucrose in TNE buffer (0·01 M Tris–HCl, pH 8·3, 0·15 M NaCl, 0·002 M ethylenediamine tetra-acetate) at 40,000 rev/min for 4 hr.

Antisera

Anti-MPMV antiserum was obtained from a rabbit injected multiple times with Nonidet P-40-disrupted MPMV grown in the NC37 cell line. About 100 μg protein in complete

Freund's adjuvant was administered s.c. into hind foot pads and back. The rabbit was boosted by monthly injections of the protein (50 μg) in incomplete Freund's adjuvant (Difco Laboratories, Detroit, Michigan). Goat anti-rabbit antiserum used for the indirect reagent was obtained from Miles Laboratories.

Preparation of tumor extracts and particulates banding in the viral density region from human leukemic cells and tumor tissues

Leukemic leukocytes and leukemic spleens were disrupted with a Silverson homogenizer. In both cases two volumes of TNE buffer were used for suspending the material. After removal of nuclei and mitochondria by low speed centrifugation (1500 ×*g* and 10,000 ×*g* 10 min each), the supernatant was used as a tumor extract and centrifuged again for further fractionation at 98,000 ×*g* on a 12-ml 20% glycerol column in TNE buffer for 1 hr. The pellet was suspended and subjected to isopycnic separation by centrifugation at 98,000 ×*g* for 16 hr through a 20–45% continuous sucrose gradient. The material in the density region between 1·15–1·17 g/ml was collected, and the particles were recovered by centrifugation at 98,000 ×*g* for 1 hr. The pellet was resuspended in 1-ml TNE buffer and used for radioimmunoassay. Protein concentrations were estimated by the Lowry method [13] based on bovine serum albumin standard.

Radioimmune precipitations

A major protein of MPMV having a molecular weight of 25,000 daltons (p25) was purified by a combination of agarose gel filtration and DEAE-cellulose column chromatography as described previously [12]. The isolated p25 was more than 95% pure as estimated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. The p25 was iodinated with ^{125}I (New England Nuclear) by the chloramine-T procedure [14]. The reaction was carried for 2–3 min at room temperature in 50 μl of 0·1 M sodium phosphate buffer at pH 7·5 containing 1 mCi ^{125}I, 5 μg protein and 5 μg chloramine-T. The reaction was stopped by addition of 8 μg (in 8 μl) of sodium metabisulfite. Free iodine was separated by passage through Sephadex G50 equilibrated with 0·1 M phosphate-buffered saline and/or dialysis against three changes of 500 ml saline at 5–10 hr intervals. The specific activity of the iodinated protein ranged from 1000–10,000 counts/min/ng.

For inhibition of indirect precipitation, the method of Kim and Dray [15] was used. The iodine-labeled p25 protein was precipitated with varying amounts of the anti-MPMV by the indirect precipitation procedure. A maximum of 72% precipitation was obtained with 0·02 μl of the antiserum. To optimize the sensitivity, the amounts of antiserum added for inhibitions of indirect precipitation were chosen to give 30–40% precipitation in the absence of inhibitor. Antibody and inhibitors were mixed and incubated for 1 hr at 37°C. In order to keep the inhibitor soluble and to prevent nonspecific adsorption of the iodinated antigen, a final concentration of 0·5% triton X-100 (Fisher Scientific Co.) was maintained for the first incubation. Iodine-labeled antigen (p25) was added then to the mixture and incubated for an additional 50 min at 37°C. The cold carrier, normal rabbit IgG (30 μg of a 14% Na_2SO_4 precipitate) and the indirect reagent, goat anti-rabbit IgG, were added sequentially. After another incubation at 37°C for 1 hr, the tubes were placed at 4°C for 16–18 hr. The precipitates were washed three times with 0·2% bovine serum albumin and 0·02% NP-40 in saline by centrifugation at 2000 rev/min for 10 min. The washed precipitates and the supernatants were counted in a Searle automatic gamma counter. The percent inhibition was normalized based on 100% precipitation without inhibitor (32–40% before normalization) and 0% precipitation without antibody.

Partial purification of cross-reactive leukemia antigen

Method for isolation of MPMV p25 [12] was applied. The viral band was prepared from human CML spleen as described in Material and Methods. After resuspension in TNE, the homogenous suspension was brought to 1% of Triton X-100 and kept in ice for 1–2 hr. Insoluble material was removed by 1 hr centrifugation at 100,000 ×*g*. The supernatant (10 ml) was loaded on a 2·5 × 70 cm agarose 0·5 M column and eluted with TNE containing 0·01% triton X-100. The third peak was pooled, diluted with water, and loaded on DE52 column equilibrated with 0·02 M phosphate pH 7·5. The fractions eluting between 0·08 M and 0·2 M NaCl were pooled, dialyzed against 2000 volume of H_2O and lyophilized to dryness.

Affinity chromatography

Monospecific antibody to MPMV p25 was coupled to CNBr activated Sepharose 4B [16]. Particles in the viral density region from 50 gm of spleen obtained from a patient with chronic ML was treated with Triton X-100 at a final concentration of 1% for 1 hr. The detergent

treated fraction was applied to the anti-p25-Sepharose column. After extensive washings (30 × volume) with TNE buffer, the protein bound to the antibody column was eluted with 0·1 M glycine-HCl buffer, pH 2·2. The eluent was dialyzed against phosphate buffered saline, then H_2O and concentrated by lyophilization.

RESULTS

Viral origin of the protein

In the initial experiments the antigen and the antiserum were prepared from virus grown in the human lymphoid cell line (NC37). It was necessary to ascertain whether the p25 antigen was derived from the virus or from the host cell. MPMV grown in NC37 and in the original Rhesus monkey mammary tumor cell line cocultivated with monkey embryo cells were compared for their ability to block the iodinated p25 protein. The viruses were both

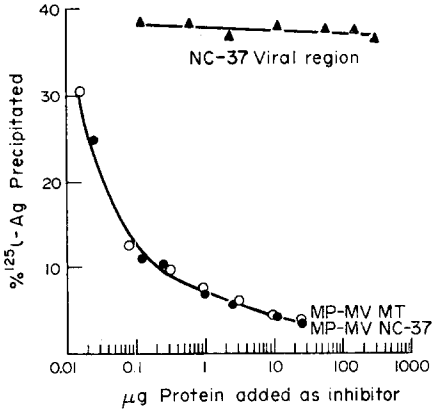

Fig. 1. Comparison of the inhibition of indirect precipitation reactions between ^{125}I-labeled MPMV p25, and anti-MPMV by MPMV grown in different hosts. The MPMV-MT (○) was grown in the original Rhesus monkey mammary tumor cells cocultivated with monkey embryo cells and the MPMV-NC37 (●) was grown in a normal human lymphocyte line, NC37. Uninfected NC37 cells were disrupted and a density region (1·15–1·19 g/ml in 20–45% sucrose density gradient) (▲) was taken for control. There was always less than a 2% difference in duplicate precipitations, and the average was recorded.

purified to the same extent and ruptured as described in Material and Methods. It is evident from Fig. 1 that the virus preparations from human and simian cell lines both yielded the same degree of inhibition through the concentration curve. As a further check, the crude extract (800 μg) and the proteins from uninfected NC37 cells, which accumulate in the viral density region (1·15–1·17 g/ml) in a sucrose gradient, were also examined. These failed to exhibit any detectable blocking ability. These results are in agreement with the studies done by others [17,18] and indicate that the labeled p25 antigen was a viral protein and not a membrane component of the host cell, NC37.

Particulate localization of cross-reacting proteins

The supernatants resulting from two successive low speed centrifugations (1500 × *g* and 10,000 × *g*, 10 min each) were tested for serological cross-reaction with p25 by inhibition of radioprecipitation. The supernatants from a variety of normal and malignant tissues including cancers of the lung, colon, rectum and breast were examined for inhibition. Only supernatants prepared from spleens and peripheral blood leukocytes (PBL) of patients with ML (acute and chronic) gave significant inhibition (> 15%) (Table 1).

Table 1. The inhibition of indirect precipitation reaction between ^{125}I-labeled MPMV p25 and anti-MPMV by post-mitochondrial supernatants of tumor tissues

Inhibitors		Protein added (μg)	Pptd. (%)	Inhibited (%)
Ca. breast	440	370	100	0
		740	93	7
Ca. breast	714	450	97	3
		675	95	5
AML spl.	1021	312	64	36
		624	66	34
CML spl.	112	520	81	19
		1040	73	27
Nor spl.	010	532	91	9
		1064	93	7
Ca. lung	176	390	95	5
		585	93	7
Ca. rectum	694	415	99	1
		830	100	0

The major cross-reactivity of the supernatant was found to be in the density of known RNA tumor viruses although this density region is not exclusive for the viral particles. This was demonstrated using the supernatant from PBL of a patient with chronic ML. The supernatant was fractionated by centrifugation through a 20–45% continuous sucrose density gradient. The fraction banding isopycnically between 1·15 and 1·17 g/ml inhibited precipitation to the greatest extent (Fig. 2). Materials in 3 other fractions showed appreciable inhibition. This may be due to the formation of the aggregation of antigens or the formation of viral core-like

particles in the high density region (> 1·20 g/ml) and the release of free or small membrane bound antigens in the lighter density regions (< 1·15 g/ml).

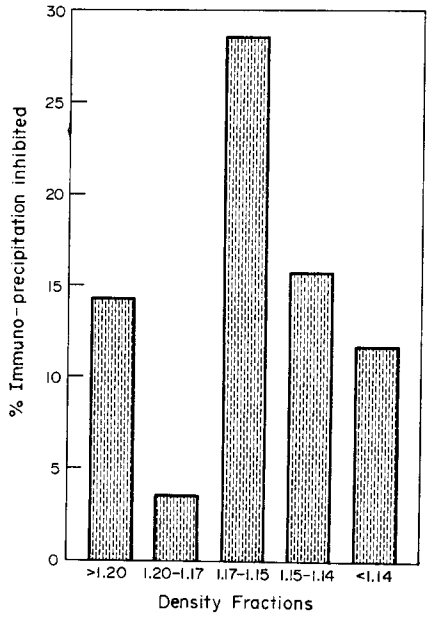

Fig. 2. Inhibition of indirect precipitation of ^{125}I-MPMV p25 by different density fractions of leukemia cell extract. The extract of WBC (500 mg) from a patient with chronic myelogenous leukemia was separated into 5 different density fractions by 20–45% sucrose density gradient centrifugation. Each fraction was pelleted by another centrifugation at 40,000 rev/min for 50 min and resuspended in 100 µl of 1% NP-40 in TNE buffer. For the inhibition of precipitation, 20 µl of each density fraction was used as an inhibitor.

Specific inhibition by antigens of myelogenous leukemia

Figure 3 illustrates some of the representative inhibition reactions of the proteins in 1·15–1·17 g/ml density region of human leukemic cells or spleen. As little as 2 µg of a preparation of this "viral density fraction" (VDF) produced 50% inhibition of precipitation. The slope of the inhibition curve obtained with VDF was similar to the curve obtained with MPMV suggesting that the leukemic tissue may contain a protein similar to MPMV p25. This very active inhibition was the highest observed, but other VDF preparations, though having less inhibitory activity, nevertheless produced similar inhibition curves.

VDF were prepared from malignant or normal tissues. Each VDF was assayed for inhibition using six different amounts ranging from 1–1000 µg. One thousand micrograms of VDF from some non-leukemic tissues inhibited by 20–25% presumably due to "non-specific interference" of the precipitin reaction caused by extremely high concentrations of proteins. However, at the 250 µg level, inhibition was only 10–15% for these tissues and, therefore, this amount of protein was used to assess the inhibition, except for VDF from PBL of patients with chronic ML, in which 120 µg were used. The results obtained with VDF prepared from 42 individuals are plotted in Fig. 4. VDF from

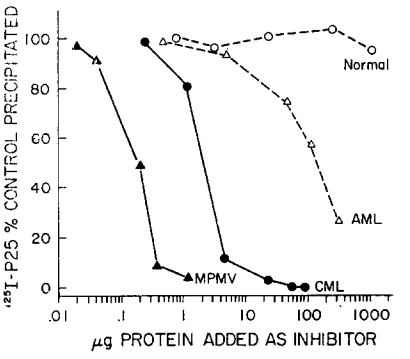

Fig. 3. Inhibition curve of immunoprecipitation of ^{125}I-MPMV p25 by various concentrations of density fractions derived from PBL of normal individuals or patients with ML. The material in the density region between 1·15–1·17 g/ml was collected, and the particles were then recovered by centrifugation at 98,000 × g for 1 hr. The pellet was resuspended in 1 ml of TNE buffer and used for radioimmunoassay. In the VDF, 80–500 µg of proteins were obtained from 1 g of starting materials.

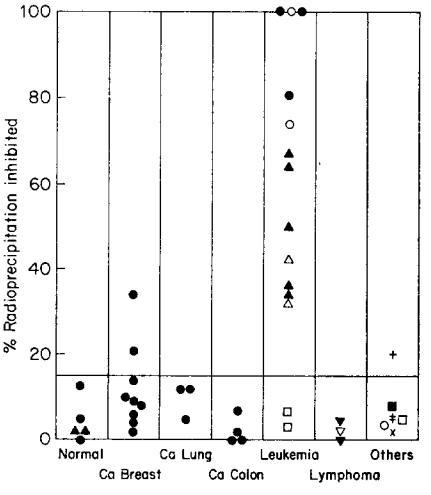

Fig. 4. Inhibition of immune precipitation of ^{125}I-p25 by proteins accumulated in the VDF of malignant or non-malignant tissues. Proteins banding on a sucrose gradient in the density region of 1·15–1·17 g/ml for ML and 1·15–1·19 for others were used for the assay. The degree of inhibition was determined at a point corresponding to 250 µg of protein. The line drawn at 15% is arbitrary to stress the significant level of the reaction. Normal PBL (●) and normal spleen (▲) for normal tissues; acute ML PBL (○), chronic ML PBL (●), acute ML spleen (△) and chronic ML spleen (▲), chronic lymphatic leukemia PBL (□) for leukemic tissues; African Burkitt's lymphoma (▼) and lymphosarcoma (▽) for lymphomas; carcinoma of the stomach (+), liposarcoma (□), Ca. pancreas (≠), Ca. bladder (○), Ca. kidney (■), and fibroadenoma (×) for others. Clinical materials from autopsies or biopsies were selected without regard to age, sex, stage or type of the malignancy or therapy.

12 of 12 patients with ML inhibited the precipitation most significantly; VDF from their PBL inhibited by 74–100%, while VDF prepared from their spleens inhibited by 32–69%. VDF obtained from two breast cancers also inhibited the precipitation significantly. None of the VDF prepared from seven other patients with breast cancer or from 20 other individuals who were either normal or had other malignancies inhibited precipitation.

Possibilities of non-specific interference by the proteins from CML leukocytes

Leukocytes from patients with ML might contain proteolytic enzymes which digest ^{125}I-p25 and cause nonspecific interference of immune precipitation by ^{125}I-p25. Experiments were done to rule out this possibility. Radiolabelled p25 was incubated for 1 hr under the same conditions used for the radioimmunoassay with 1 mg of the VDF from PBL of a patient with chronic ML. Fragmentation of ^{125}I-p25 was estimated by an electrophoresis of the mixture in 1% SDS-7·5% polyacrylamide and precipitability in 10% trichloroacetic acid. Insignificant levels of the radioactivity over background of free ^{125}I (10%) were observed either in the low molecular weight region of SDS gel or in the supernatant of 10% trichloroacetic acid solution. In addition, the same leukemic materials which were inhibitory to the precipitation of ^{125}I-p25 were not inhibitory to the precipitations of ^{125}I-Rauscher leukemia virus p30, ^{125}I-mouse mammary tumor virus gp52 and ^{125}I-Avian myeloblastosis virus p27. A representative result is shown in Fig. 5. Since no immunological cross-reaction is detectable among MPMV p25, Rauscher leukemia virus p30 and mouse mammary tumor virus gp52 [10,17], it was concluded that the inhibition is immunologically specific. Taken together, these results indicate that significant proteolysis has not occurred, and the inhibition is specific to p25.

Partial purification and characterization of the leukemia antigen

To investigate the nature of the cross-reaction between MPMV p25 and an antigen from human ML, partial purification and characterization of the leukemic antigen were performed. The VDF from PBL of a patient with chronic ML was partially purified by agarose-gel filtration. The enriched antigen was further separated by disc-gel electrophoresis in Tris-glycine buffer, pH 8·4, to determine relative net electrical charge to p25. The degree of cross-reactivity was estimated by inhibition of the radioimmune precipitation of ^{125}I-p25 with the eluent proteins from 3 mm sections of the gel. The peak of the cross-reactivity was found in the gel fractions to be in the same positions as MPMV p25, indicating similar net electrical charge to p25 (Fig. 6A). The peak of reactivity was relatively broad. This could be explained by overloading the gels with the sample (1–2 mg/gel of 6 mm diameter). Overloading was necessary due to the low efficiency of elution and the minor population of cross-reacting protein in the sample. However, this does not exclude the possibility of the presence of more than one cross-reacting protein with similar net electrical charges.

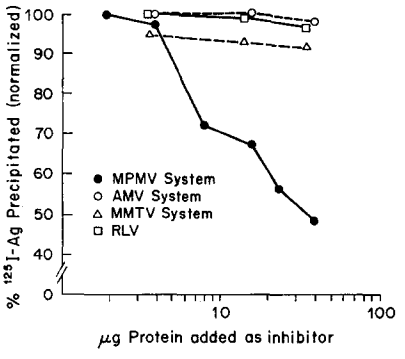

Fig. 5. Immunological specific inhibition of MPMV p25 anti-MPMV by partially purified human protein. Partially purified leukemia antigen was applied to 4 different radioimmuno-precipitation systems. The ^{125}I-labeled antigen–antibody systems used were: (a) AMV p27 (100,000 counts/min/ng) and anti-AMV p27 giving 32% precipitation; (b) RLV p30 (10,000 counts/min/ng) and anti-RLV p30 giving 47% precipitation; (c) ^{125}I-MMTV GP52 (8000 counts/min/ng) and anti-MMTV giving 37% precipitation, and (d) ^{125}I-MPMV-p25 (20,000 counts/min/ng) and anti-MPMV giving 51% precipitation. One μg of AMV, RLV, MMTV or MPMV inhibited completely homologous radioimmunoassay systems respectively. Increasing amounts of human leukemic protein were added to each of the assays. The data were normalized to 100% precipitation without inhibitors with the fixed amount of antiserum.

Furthermore, the cross-reacting protein was partially isolated using an immunoabsorbent column of rabbit anti-MPMV p25 (Fig. 6B). The eluate showed a major peak of molecular weight 26,000 daltons in SDS gel electrophoresis which is very close to MPMV p25 (Fig. 6B). As a minor peak, a protein with molecular weight 12,000 daltons was also found in the eluate. It might be due to dual specificity of the antibodies coupled to the Sepharose although the antiserum gave a single precipitin line against NP-40-disrupted MPMV in agar gel double diffusion. This possibility is supported by the electrophoresis pattern of eluate from

the column which had been applied with NP-40 disrupted MPMV. This result may suggest the presence of another antigen, p12, in human leukemic materials which is cross-reactive to MPMV p12. The purified human protein retained partial inhibitory activity for the radioimmune precipitation (10 μg was required for the 50% inhibition) of MPMV system. However, it did not inhibit the other radioimmune precipitation systems of viral proteins such as RLV p30-anti-p30. The failure to obtain 100% recovery of the activity may be due to the extensive manipulation and acid treatment of the material. When the leukemic material was applied to a column of Sepharose 4B coupled with normal rabbit IgG, no detectable protein was recovered in the eluate.

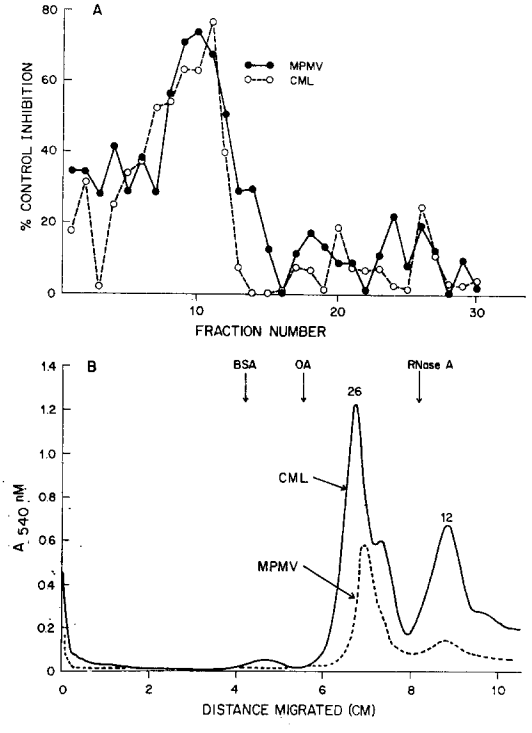

Fig. 6. Characterization of the human myelogenous leukemia antigen.

A. Similar net electrical charge to MPMV p25.

The particles from CML spleen which accumulated in the 1·16 g/ml region of a sucrose density gradient were disrupted with 1% Triton X-100 for 1 hr. The detergent-disrupted particles were fractionated by gel filtration on an agarose (A 0·5 M), and fractions showing greatest inhibition in the radioimmunoassay were pooled, desalted and concentrated. This preparation was further separated by disc-gel electrophoresis in Tris-glycine buffer, pH 8·4. The gel was cut into 3 mm fractions which were ground to 400 μl of TNE buffer, 200 μl of the resulting supernatant was used for inhibition of the radio-precipitation. This same procedure was applied to MPMV for a control reference.

B. Similar molecular weight to MPMV p25.

The detergent disrupted particles (prepared as above) were applied to the anti-p25-Sepharose column. After extensive washings (30 × volume) with TNE buffer, the protein bound to the antibody-column was eluted with 0·1 M glycine-HCl buffer, pH 2·2. The eluate was dialyzed extensively against phosphate buffered saline and H_2O, and then lyophilized. The concentrated material was analyzed by 1% SDS-7·5% polyacrylamide gel electrophoresis. Bovine serum albumin, ovalbumin and RNase A were used as references for molecular weights.

DISCUSSION

In the present studies, the existence of a cross-reacting antigen in the leukocytes of patients with myelogenous leukemia to the major protein (p25) of MPMV has been demonstrated. It is a striking fact that 12 out of 12 of the myelogenous tissues (PBL and spleens) were positive for the blocking protein. It is of interest that an antigen similar to the MPMV p27 (or p25) has been found in some human malignant breast tumors [19], and this may account for the two positive samples out of 9 viral density fractions of malignant breast tissues. Although its relation to the disease is unknown, MPMV apparently originated from a mammary tumor in a monkey [1].

Under the circumstances, it is surprising that an antigenic relationship was found between the p25 of MPMV and an antigen found in the human myelogenous leukemias. However, MPMV-like particles have been identified in human cell lines derived from patients with leukemia or other malignancies [7–9]. Relatively high levels of antibodies to the MPMV p25 were found in plasmas from some normal individuals and most patients with myelogenous leukemia [12]. Therefore, infection with the same or similar virus might be widespread and be associated with both leukemia and carcinoma of the breast.

No significant precipitation of ^{125}I-p25 was observed either by anti-gs3 (RLV) or by anti-MMTV (unpublished data). These results agree with the observations that MPMV does not cross-react serologically with other mammalian B- or C-type viral proteins [3, 10, 17]. Thus, the absence of interactions between MPMV-p25 and Rauscher leukemia virus or simian sarcoma virus makes it unlikely that we are dealing with antigens previously reported in normal and some human tumor tissues [20–23].

A number of experiments were done to exclude the possibilities of proteolysis which might interfere with immune precipitations nonspecifically. It is very unlikely that the inhibition of radioimmune precipitation had occurred by proteolysis for the following reasons: (1) no detectable fragmentation of ^{125}I-p25 was found by an active VDF (SDS

gel electrophoresis and 10% trichloroacetic acid precipitation); (2) the leukemic material which gave positive reaction did not inhibit the radioimmune precipitations of other RNA tumor viral proteins; (3) the leukemia antigen purified by specific immunoabsorbent column retained the inhibitory activity for the immune precipitation of ^{125}I-p25; (4) the net electrical charge and molecular weight of the leukemia antigen are very similar to those of MPMV p25.

On the basis of the present studies, it appears that the cross-reacting antigen obtained from cells from patients with ML is very similar to an antigen of the MPMV p25. The presence of antibody to the MPMV p25 in the plasmas of patients with myelogenous leukemia strongly supports this possibility [12]. Using protease inhibitors to rule out the possibility of protease is not appropriate for an immunological system. In most cases, the protease inhibitors modify active sites of enzymes and the use of such inhibitors might affect antigenicity and create uninterpretable artifacts. Although it is extremely unlikely, a possibility of a protease specific for MPMV p25 is not completely excluded. Even if it is assumed that proteolysis occurs, it would be most remarkable that the proteolysis is so specific for an enzyme present exclusively in VDF of ML materials. The inhibition appears to be so specific to myelogenous leukemia that it might provide valuable clinical information. For example, it is of interest that in preliminary studies leukocytes obtained from two patients in remission from myelogenous leukemia showed no detectable inhibition. Thus, the assay may be useful for following the course or determining the prognosis of myelogenous leukemia.

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Inhibition of Chemical Carcinogenesis: Increased Activity of Soluble RNA Polymerase in the Liver of Rats Protected Against 3'MeDAB Hepatocarcinogenesis by Dietary Chloramphenicol*

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Abstract—*Soluble RNA polymerases (nucleotidyl transferases, EC 2.7.7.6) were isolated from liver nuclei of rats that had been pair-fed diets containing the hepatocarcinogen 3'methyl-4-dimethylaminoazobenzene (3'MeDAB, 0·06%) either alone or in combination with chloramphenicol (CAP, 2%), an inhibitor of azo dye carcinogenesis. DEAE Sephadex A-25 chromatography of crude nuclear extracts (fraction IV protein) revealed 2 major peaks of enzyme activity which were equated with RNA polymerases I and II on the basis of order of elution and sensitivity to α-amanitin. The specific activities of both enzymes, in particular RNA polymerase I, were significantly increased after 4 days on diets containing CAP or both 3'MeDAB and CAP. Rats protected from 3'MeDAB carcinogenesis by CAP showed the greatest increase, which exceeded that in the group fed dye alone by 292% for polymerase I and 116% for polymerase II. Increases in both RNA polymerase activities were probably not a consequence of altered affinity for the substrate UTP. RNA polymerase activity was not altered by feeding the 3'MeDAB diet for 4 days, but after 10 days there was a 340% increase in the specific activity of enzyme I and a 14% increase in the specific activity of enzyme II relative to levels in control rats. The total nuclear protein content was significantly increased in rats fed 3'MeDAB or both 3'MeDAB and CAP for 4 days. Crude polymerase extracts contained 4–6% of the total nuclear protein and 83–87% of the RNA polymerase activity present in isolated nuclei. There was a significant increase in the amount of nuclear sap protein extracted after feeding 3'MeDAB for 4 days. Differences in nuclear RNA synthesis between experimental groups were insensitive to presacrifice starvation and altered feeding and lighting schedules; neither were they due to differential losses of RNA polymerases during the nuclear isolation procedure or to preferential extraction of these enzymes from isolated nuclei in the various groups. We conclude that the differences in liver nuclear RNA synthesis between 3'MeDAB-fed rats and rats protected against 3'MeDAB-induced hepatocarcinogenesis by concurrent CAP administration are at least partly due to differences in the activity and/or amount of soluble RNA polymerases.*

INTRODUCTION

CHLORAMPHENICOL (CAP) inhibits azo dye hepatocarcinogenesis in the rat [1, 2]. The liver RNA/DNA ratio of protected rats is normal, in contrast with that of rats fed an azo dye, in which the ratio is significantly depressed [3]. This effect of CAP could be relevant to the protective action, for similar levels of protein-bound carcinogen were found in the livers of both dye-fed and CAP-protected rats [3], indicating that CAP administration probably

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does not significantly affect the amount of carcinogen reaching the target tissue. The difference in liver RNA/DNA ratio between azo dye-fed and CAP-protected rats is due to differences in the amount of liver RNA [3]. Furthermore, there is evidence that this effect is a consequence of an increased rate of nuclear RNA synthesis, rather than slower degradation of total liver RNA [4, 5]. Alterations in nuclear RNA synthesis can be due to changes in the template activity of DNA and chromatin or in the activity and amount of the RNA polymerases (nucleotidyl transferases, EC 2.7.7.6). Methods describing the isolation of these RNA polymerases in high yield are now available [6–8] and in the present report, we describe some properties of purified RNA polymerases from the livers of rats which had been fed diets containing the azo dye 3′methyl-4-dimethylaminoazobenzene (3′MeDAB) either alone or in combination with CAP. The specific activities of both RNA polymerases I and II, especially the former, were found to be significantly increased in CAP-protected rats as compared with rats fed only 3′MeDAB.

The differences in soluble RNA polymerase activity between carcinogen-fed and CAP-protected rats described here have been shown not to be due to differences in the efficiency of extraction of soluble polymerase from isolated nuclei. Neither are they due to differential leaching of the enzyme activity from nuclei during the isolation procedure. Altered feeding and lighting schedules did not affect the pattern of our previous results [4], where administration of a diet containing 3′MeDAB and CAP caused a significant increase in RNA synthesis in isolated rat liver nuclei as compared with rats pair-fed a diet containing only 3′MeDAB.

MATERIAL AND METHODS

Animals and diets

Male Sprague–Dawley rats (a total of 96 of approx wt 145 g) random bred from local departmental stock were used in all experiments. They were housed in individual cages and pair-fed either a control diet or diets containing 2% CAP, 0·06% 3′MeDAB and 0·06% 3′MeDAB plus 2% CAP for 4 or 10 days as indicated in the Table and Figure legends.

Details of the diet preparation and pair-feeding procedure have been described previously [3]. Except where specified otherwise, the rats had continuous access to the diets until the time of sacrifice. At the conclusion of each experiment, the rats were killed by exsanguination under light ether anaesthesia between 09.00 and 10.00 hr.

Source of materials

The following reagents were purchased from the Sigma Chemical Co., St. Louis, MO: ATP (disodium salt), CTP (disodium salt), GTP (trisodium salt), UTP (tetrasodium salt), spermine diphosphate, dithioerythritol (DTE), dithiothreitol (DTT), ribonuclease A (type 1A, from bovine pancreas), protease (type VI, from *S. griseus*) and calf thymus DNA (type 1, highly polymerized). Uridine-4-^{14}C-5′-triphosphate (specific activity 43 mCi/m-mole) and uridine-5,6-^{3}H-5′-triphosphate (48 Ci/m-mole) were obtained from the Radiochemical Centre, Amersham, Bucks. and were periodically checked for radiopurity by paper chromatography [9]. α-Amanitin was purchased from Calbiochem, San Diego, Cal. and manufactured by C. H. Boehringer Ingelheim. Eastman Kodak Co., Rochester, N.Y. supplied the N, N-methylene bisacrylamide and N, N, N′, N′- tetramethylethylenediamine (TEMED) while cellulose casing dialysis tubing, size 18/32, was obtained from the Visking Co., Chicago, Ill. Crystalline bovine serum albumin (BSA), Cohn fraction V, was purchased from the Commonwealth Serum Laboratories, Melbourne and DEAE Sephadex A-25 from Pharmacia, Uppsala. 3′MeDAB (m.p. 119° C) was synthesized as described by Giese *et al.* [10] and CAP (D(–) *threo* isomer) was a gift from Parke, Davis and Co., Caringbah. Coomassie brilliant blue FF (1434) was obtained from E. Gurr, Ltd., Bucks. All other chemicals used were of analytical reagent grade and solutions were prepared with metal-distilled water which had been passed through an Elgastat deionizer.

Rat liver histone was prepared according to the recommendations of Hnilica [11]. The histone content of our preparation was determined by the method of Mirsky and Pollister [12] as described by Hnilica [11] and the

$$E\,\frac{354\text{ nm}}{1\text{ mg/ml}}$$

was found to be 0·785 as compared with a literature value of 0·800 [11].

The procedure followed for the preparation of rat liver DNA was basically that of Marmur [13] and has been described elsewhere [14]. The DNA obtained was dissolved in phosphate-citrate buffer (0·01 M phosphate/0·001 M citrate, pH 7·5) at a final concentration of 1·462 mg/ml and stored at −20° C prior to use. Analytical ultracentrifugation of a sample of

this preparation revealed a single polydisperse peak with a sedimentation coefficient of 21·3 ± 2·6 (S.D.). In contrast with the findings of a recent report [15], there was no detectable DNA nuclease activity in the commercial ribonuclease and protease preparations used during the isolation of the DNA. Furthermore, the buoyant density in CsCl [16] of a sample of commercial calf thymus DNA was unaltered if it was subjected to the DNA isolation procedure. Calf thymus DNA also showed a lack of hyperchromicity [17] in the presence of either the ribonuclease and/or the protease. DNA nuclease activity was determined by the procedure recommended by the Sigma Chemical Co., St. Louis, MO after heating the ribonuclease at 90° C for 10 min and autodigesting the protease at 37° C for 2 hr.

Isolation of nuclei

The basic technique of Blobel and Potter [18] was routinely used for isolating nuclei prior to solubilization of the DNA-dependent RNA polymerases.

When studies were made of the nuclear protein composition and also in the controlled feeding experiments, nuclei were isolated by a modification [4] of the method of Widnell and Tata [19], for more liver samples could be processed by using this procedure. The recovery of liver DNA in these nuclear preparations varied between 43 and 47%. The large scale nuclear isolation procedure of Read and Mauritzen [20] was used when nuclei were required for the preparation of rat liver histone standard.

When estimations were made of the amount of "free" RNA polymerase activity present in nuclear preparations, a comparison was made of nuclei isolated by the Blobel and Potter technique with those isolated by using the procedure of Yu [21] which we modified by omitting the perfusion step and substituting $MgCl_2$ for $CaCl_2$ in the homogenizing medium. The nuclei obtained had an RNA/DNA ratio of 0·199 as compared with a value of 0·184 for nuclei prepared from an identical liver sample by the Blobel and Potter procedure. Nuclei isolated by either the Blobel and Potter technique described above or the modified Widnell and Tata procedure were comparable with respect to percent recovery of total homogenate DNA, RNA/DNA ratio and capacity for RNA synthesis. Total homogenate DNA and RNA were determined using the procedure described by Munro and Fleck [22], while nuclear DNA and RNA were estimated as described by Blobel and Potter [23]. All nuclear pellets were resuspended in 4 ml of 0·25 M sucrose containing 1 mM $MgCl_2$.

Solubilization of RNA polymerases

Aliquots of the respective nuclear suspensions were taken for the estimation of RNA synthesis, then the residual portions were centrifuged at 750 g for 10 min to pellet the nuclei prior to extraction of the RNA polymerase. In our experience, the method of Jacob *et al.* [8] was found to give high yields of active enzyme and was used routinely. The dialysed protein preparation (fraction IV protein) did not require clarification by centrifugation as no precipitate was evident. A portion of the fraction IV protein was immediately assayed for DNA-dependent RNA polymerase activity and the remainder subjected to ion exchange chromatography within 5 hr of dialysis.

Ion exchange chromatography

DEAE Sephadex A-25 was prepared as described by Saunders *et al.* [24]. Fraction IV protein from the various rats (1–8 mg in 2·5–5·5 ml) was applied to columns (0·9 × 12 cm) and was washed into the gel with 0·05 M $(NH_4)_2SO_4$ in a solution comprising 0·05 M Tris-HCl (pH 7·9), 25% (v/v) glycerol, 5 mM $MgCl_2$, 0·1 mM ethylenediaminetetraacetate (EDTA) and 0·5 mM dithiothreitol (TGMED, 20 ml). The enzymes were then eluted with a linear gradient of 0·05–1·2 M $(NH_4)_2SO_4$ in TGMED (70 + 70 ml). A total of 110 fractions (1 ml) were collected, commencing at the time of sample application. The flow rates of the columns were *ca* 35 ml/hr.

The concentration of $(NH_4)_2SO_4$ in the individual fractions was determined by either the phenol-hypochlorite reaction for ammonia [25] or by measurement of the conductivity (Philips conductivity-measuring bridge PR 9500) or resistance (Avo multimeter) of the solution. The phenol–hypochlorite method required that aliquots (100 µl) of the fractions be diluted (1 part in 20,000 parts of water) prior to analysis, while conductivity or resistance measurements were made on undiluted eluate. The $(NH_4)_2SO_4$ concentration of the respective samples was estimated from a graph of standard values obtained by analysis of solutions of known concentrations of $(NH_4)_2SO_4$ in TGMED. Assays of RNA polymerase activity in the fractions were always performed within 12 hr of chromatography.

Assay of RNA synthesis

Nuclear RNA synthesis was estimated by the method of Widnell and Tata [26] as detailed

in a previous report [4]. RNA polymerase activity in fraction IV protein was routinely determined by the method of Jacob *et al.* [8]. The fractions obtained after DEAE Sephadex chromatography were also assayed for RNA polymerase activity by the method described by Jacob *et al.* [8] for the Mg^{2+}-dependent activity of fraction IV protein, except that $MnCl_2$ (1·5 μmole) was also included in the incubation medium, the amount of unlabelled UTP used was 0·015 μmole and ^{3}H-UTP (1 μCi) was used as the radioactive precursor. In a pilot study, α-amanitin (0·08μg in 50μl) was added to the media immediately prior to initiation in RNA synthesis assays on samples representative of the various preparations.

The various incubations were routinely terminated by the addition of ice-cold 0·3 M $HClO_4$ (1 ml) and the precipitates collected by centrifugation. They were then washed either twice (^{14}C-UTP incubations) or five times (^{3}H-UTP incubations) with 0·2 M $HClO_4$ (3 ml) and hydrolyzed for 15 min at 37°C with 0·3 M KOH (1 ml). The volume was made up to 2·9 ml with deionized water and a portion (1 ml) of this solution neutralized with conc. HCl (100 μl); an aliquot (7·5 ml) of scintillation mixture comprising 2:1 toluene/Triton X-100 containing 4 g PPO/l. was then added and the radioactivity of the samples determined.

The above-mentioned procedure for estimating incorporation of radioactivity into RNA gave results which were comparable with those of the Blobel and Potter procedure [23]. All radioactivity measurements were made with a Packard model 3314 liquid scintillation spectrometer. Addition of ^{14}C-toluene or ^{3}H-toluene internal standards revealed that the counting efficiency was 80% for ^{14}C and 29% for ^{3}H.

Polyacrylamide gel electrophoresis

Chromatographic fractions which contained peaks of RNA polymerase activity were pooled, stored at −70° C for 3 months and then lyophilized. Each sample was resuspended in 50 μl of a buffer (identical except for the omission of sodium dodecyl sulphate (SDS) with that described by Weaver *et al.* [27]); 10–15 μl of the resulting solutions were then layered over 5% acrylamide gels (pH 8·5). The gels were prepared by the method of Shapiro *et al.* [28] except that once again, SDS was omitted. Electrophoresis was performed under nondenaturing conditions for 3 hr at a constant current of 8 mA/gel in a Quickfit polyacrylamide gel electrophoresis apparatus, type DO 95270/1. The separated proteins were stained with Coomassie brilliant blue FF (1434) as described by Weber and Osborn [29].

Extraction of nuclear proteins

The protein composition of nuclear preparations was determined both before and after solubilization of the DNA-dependent RNA polymerases. The basic procedure used for the fractionation of the nuclear proteins was that of Teng *et al.* [30] but the sap proteins and acidic proteins were extracted by the methods of Hnilica [11] and Elgin and Bonner [31] respectively.

Protein estimation

Total nuclear protein was determined by the microbiuret method of Itzhaki and Gill [32] and the protein content of the homogenate by the biuret method as described by Cleland and Slater [33]. The protein content of fraction IV protein was determined by measuring A_{260nm}/A_{280nm} [34], while that of the nuclear protein fractions and the column effluent was determined by measuring A_{215nm}/A_{225nm} [35, 36]. The histone content of the column effluent was measured by the method of Mirsky and Pollister [12] as described by Hnilica [11].

Statistical analysis

Statistical analysis was performed with the two-tailed Student's *t*-test unless otherwise indicated; a *P* value of less than 0·05 was taken as being significant.

RESULTS

Effects of controlled food access and lighting on RNA synthesis in isolated nuclei

In agreement with the results of a previous study [4] rats protected against 3′MeDAB-induced hepatocarcinogenesis by the inclusion of CAP in the diet, exhibited a significantly increased level of nuclear RNA synthesis as compared with rats fed 3′MeDAB alone (results not shown). The effect was apparent within 4 days of commencing the diets and was most pronounced in the Mg^{2+}-stimulated assay, which predominantly measures ribosomal RNA synthesis [37].

There are recent reports, however, that both Mg^{2+}-stimulated and $Mn^{2+}/(NH_4)_2SO_4$-stimulated nuclear RNA synthetic activities exhibit a diurnal rhythm [38, 39] and that these periodic fluctuations in activity are related to food intake [38]. The CAP-containing

diets are unpalatable and furthermore, there is evidence that the feeding pattern of rats is altered by the administration of a hepatocarcinogen in the diet [40]. The differences in nuclear RNA synthesis between dye-fed and CAP-protected rats might therefore be an artifact. This could arise from different feeding patterns in rats eating the respective diets, for the animals, although pair-fed, were allowed continuous access to the food. RNA synthesis in isolated liver nuclei was therefore compared in rats pair-fed diets containing azo dye and/or CAP by the conventional procedure [3], in rats pair-fed and starved 16 hr prior to sacrifice, and in rats subjected to the "8 + 16" feeding and "12 + 12" inverted and displaced lighting schedules of Potter *et al.* [41] as described by Barbiroli *et al.* [38]. These rats were also starved for 16 hr before sacrifice. The results of this experiment indicated that the differences in nuclear RNA synthesis between dye-fed and CAP-protected rats were not a consequence of altered feeding patterns induced by the diets.

Comparison of methods for extraction of RNA polymerase activity

Initially, the procedures described by Roeder and Rutter [6] and Jacob *et al.* [8] for the solubilization of rat liver RNA polymerases were compared with respect to the yield of active enzyme obtained. There are several difficulties involved in quantitating this parameter, perhaps the most serious being that the enzyme present in isolated nuclei reads from an endogenous chromatin template, while the solubilized enzyme is routinely assayed with free DNA, usually of a relatively low mol. wt. We therefore measured both the extracted activity and the residual activity in the nucleus after the extraction procedure, as recommended by Jacob [42]. The results showed that the amount of activity extracted agreed quite well with the level of activity remaining in the nucleus after extraction; on this basis 82·6% of the Mg^{2+}-stimulated activity and 86·6% of the $Mn^{2+}/(NH_4)_2SO_4$-stimulated activity were extracted. The procedure advocated by Jacob *et al.* [8] was also, in our hands, found to be more efficient than that of Roeder and Rutter [6] for solubilizing rat liver RNA polymerases. This finding could be due in part to the absence of a sonication step in the method of Jacob *et al.* [8], for sonication has been shown by others to decrease the yield of active enzyme [21, 43]. Furthermore, the procedure of Jacob *et al.* [8] requires that the dissociated polymerases be separated from the chromatin by centrifugation at 10° C, this temperature presumably being necessary to prevent reaggregation of enzyme and template [44]. In contrast, in the method described by Roeder and Rutter [6], the centrifugation step is performed at 0–4° C. These conditions might permit reaggregation to occur with a consequent decrease in the yield of soluble RNA polymerases.

Effects of diets containing 3′MeDAB and/or CAP on extractable RNA polymerase activity

Rats protected against 3′MeDAB-induced carcinogenesis by concurrent administration of CAP possessed significantly greater ($P < 0{\cdot}01$) total amounts of crude liver RNA polymerase activity, as estimated in fraction IV protein preparations, than rats fed 3′MeDAB alone (Table 1). However, there were increases in the amounts of total crude RNA polymerase activity extractable from the livers of all rats fed the experimental diets (CAP, 3′MeDAB, or both 3′MeDAB plus CAP). The same pattern of results was apparent for both the Mg^{2+}-stimulated and the $Mn^{2+}/(NH_4)_2SO_4$-stimulated assays, but were more pronounced in the former. When RNA polymerases I and II were estimated separately following DEAE Sephadex chromatography of fraction IV protein preparations, the alterations in the activity of these enzymes that had been noted in the crude preparations were confirmed. In particular, the protected rats, which had been fed both 3′MeDAB plus CAP, had much more extractable RNA polymerase I and II activity than those fed 3′MeDAB alone ($P < 0{\cdot}05$).

After 10 days on the diets, rats pair-fed the diet containing 3′MeDAB still showed an increase in extractable enzyme activity (Table 1). It is interesting to note that greater levels of enzyme activity were extracted from both control and 3′MeDAB-treated rats after 10 days of pair feeding than after only 4 days. These results are in contrast with data on the RNA synthetic activity in whole nuclei in these rats, which in general decreases rather than increases during this time interval [4]. The state of semi-starvation induced by the pair feeding regimens might result in altered nuclear structure and facilitate extraction of the enzyme activity; alternatively, there could be absolute increases in the amount of enzyme protein. The decreased levels of nuclear RNA synthesis, despite an increase in the amount of extractable enzyme, might be due to restriction of the chromatin template in semistarved rats [45].

Table 1. Effect of pair feeding a control diet and diets containing 2% CAP, 0·06% 3′MeDAB and 0·06% 3′MeDAB plus 2% CAP to rats for 4 or 10 days on the total amount of extractable RNA polymerase activity in the livers

Diet	4 days on diet				10 days on diet			
	Fraction IV protein		Column purified enzyme		Fraction IV protein		Column purified enzyme	
	Mg^{2+}-stimulated activity	$Mn^{2+}/(NH_4)_2SO_4$-stimulated activity	Polymerase I	Polymerase II	Mg^{2+}-stimulated activity	$Mn^{2+}/(NH_4)_2SO_4$-stimulated activity	Polymerase I	Polymerase II
Control	643 ± 59 (6)	806 ± 78 (6)	626 ± 60 (5)	822 ± 80 (5)	1558 ± 616 (3)	3052 ± 2169 (3)	1722 ± 1067 (3)	2905 ± 1954 (3)
CAP	924 ± 87* (3)	1121 ± 46* (3)	928 ± 71* (3)	1130 ± 47* (3)	—	—	—	—
3′MeDAB	1254 ± 375* (8)	1271 ± 271* (8)	1145 ± 495‡ (5)	1174 ± 374 (5)	2407 ± 1022† (3)	4627 ± 3245† (3)	2431 ± 1271† (3)	4242 ± 2307† (3)
3′MeDAB plus CAP	3214 ± 616* (5)	2327 ± 638* (5)	3261 ± 810* (3)	2165 ± 721* (3)	—	—	—	—

The results are expressed as pmole UTP incorporated/liver/100 g body wt and represent the mean ± S.D. of estimations on the number of rats shown in parentheses. A total of 28 rats (mean wt ± S.D.; 147 ± 8 g) were used in these experiments. The data shown for the column-purified enzymes (polymerases I and II) represent the areas under the peaks (I and II) that were obtained on DEAE Sephadex A-25 chromatography. These values have been corrected for losses occurring prior to and during chromatography by using the percent recovery data given in Table 7. The corresponding data for the protein content of these fractions are given in Table 4.

*Significantly different from the respective control group ($P < 0·01$).

†Significantly different from the respective control group ($P < 0·01$, using the one-tailed Student's *t*-test).

‡Significantly different from the respective control group ($P < 0·05$).

Effects of diets containing 3'MeDAB and/or CAP on loss of RNA polymerase during nuclear isolation and on efficiency of extraction of the enzymes from isolated nuclei

Differences in the amount of RNA polymerase solubilized from liver nuclei such as those shown in Table 1 could be a result of differential enzyme extraction from nuclei of rats fed the various experimental diets. To test this hypothesis, the residual RNA synthetic activity was measured in nuclei isolated from rats pair-fed the control and experimental diets. Levels of residual activity as measured by both the Mg^{2+}-stimulated and $Mn^{2+}/(NH_4)_2SO_4$-stimulated assays were roughly similar. This finding suggests that the differences observed between the groups in the amounts of extractable RNA polymerase activity are not due to altered solubilization of these enzymes from the nuclei of the experimental rats.

Selective leaching of RNA polymerases during the nuclear isolation procedure could also account for differences in the amounts of enzyme activity extractable from nuclei of the livers of rats fed the control and experimental diets. Yu [21] has recently reported that as much as 50% of the total nuclear RNA polymerase activity in normal rat liver may exist in the form of 'free' enzyme which is easily lost when the isolation procedure involves the use of an isotonic buffer medium. We therefore compared nuclei isolated directly in hypertonic sucrose [21] with those isolated by the Blobel and Potter procedure [18]. Levels of Mg^{2+}-stimulated RNA synthesis were roughly comparable in nuclei isolated by either procedure from rats fed the control or experimental diets. However, when exogenous DNA was added, a much greater increase in measurable enzyme activity was noted in nuclei isolated in hypertonic sucrose (Fig. 1). These findings indicated that, in agreement with Yu [21], there is measurable 'free' RNA polymerase activity in rat liver nuclei and that there is more of this 'free' enzyme if the nuclei are isolated in hypertonic sucrose. However, the relative differences in Mg^{2+}-stimulated RNA synthetic activity between nuclei isolated from rats fed the control and experimental diets were even more pronounced when measurements were made on nuclei isolated in hypertonic sucrose and in the presence of exogenous DNA. These findings exclude the selective leaching of RNA polymerase activity during nuclear isolation as an explanation of the differences in extractable RNA polymerase I activity in the variously-treated rats.

A basically similar pattern of results to those obtained for the Mg^{2+}-stimulated activity was observed if $Mn^{2+}/(NH_4)_2SO_4$-stimulated RNA synthesis was measured in nuclei isolated by the Yu [21] and Blobel and Potter [18] procedures. However in this instance, a greater amount of 'free' enzyme activity was noted

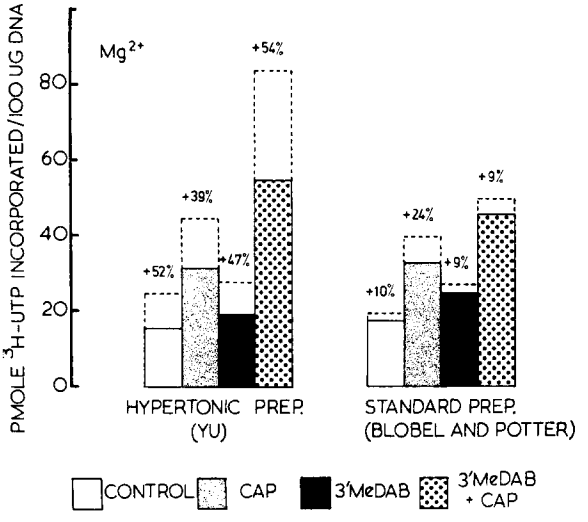

Fig. 1. Effect of the nuclear isolation procedure used on the level of Mg^{2+}-stimulated RNA synthesis in isolated liver nuclei from rats pair-fed either a control diet or diets containing 2% CAP, 0·06% 3'MeDAB or both 0·06% 3'MeDAB plus 2% CAP for 4 days. Pooled liver samples from 4 rats in each group were divided into two equal portions and nuclei were isolated from each of these according to procedures described by Yu [21] or Blobel and Potter [18] and outlined in 'Material and Methods'. A total of 16 rats (mean wt ± S.D.; 134 ± 11 g) were used. The percentage increases in incorporation indicated by the dotted lines were observed if exogenous rat liver DNA (84 μg) was added prior to the incubation; these increases represent the amount of 'free' RNA polymerase activity present in the nuclei. All incubations were performed in triplicate.

in the nuclei isolated in isotonic medium (Fig. 2), and the level of 'free' enzyme activity measured was similar regardless of the procedure used for nuclear isolation. These results indicate that the $Mn^{2+}/(NH_4)_2SO_4$-stimulated activity is more resistant to leaching during the isolation of nuclei. The relative differences in $Mn^{2+}/(NH_4)_2SO_4$-stimulated RNA synthetic activity between nuclei isolated from rats fed the control and experimental diets were, as in the instance of the Mg^{2+}-stimulated activity, more pronounced when exogenous DNA was added to the assays. Selective leaching of RNA polymerase activity during nuclear isolation therefore could also not explain differences in extractable RNA polymerase II activity in the variously-treated rats.

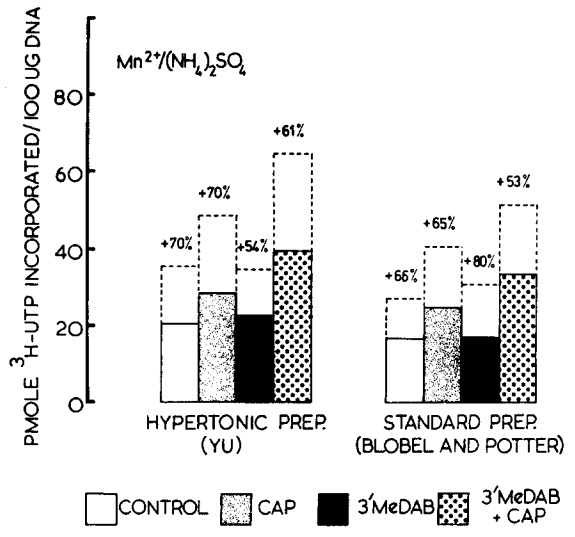

Fig. 2. Effect of the nuclear isolation procedure used on the level of $Mn^{2+}/(NH_4)_2SO_4$-stimulated RNA synthesis in isolated liver nuclei from rats pair-fed either a control diet or diets containing 2% CAP, 0·06% 3'MeDAB or both 0·06% 3'MeDAB plus 2% CAP for 4 days. Pooled liver samples from 4 rats in each group were divided into two equal portions and nuclei were isolated from each of these according to procedures described by Yu [21] or Blobel and Potter [18] and outlined in 'Material and Methods'. A total of 16 rats (mean wt ± S.D.; 134 ± 11 g) were used. The percentage increases in incorporation indicated by the dotted lines were observed if exogenous rat liver DNA (84 μg) was added prior to the incubation; these increases represent the amount of 'free' RNA polymerase activity present in the nuclei. All incubations were performed in triplicate.

Characteristics of nuclear proteins extracted with the RNA polymerases

There were increases in total liver nuclear DNA, RNA and protein in rats pair-fed the experimental diets for 4 days (Table 2). The increases were significant for DNA in rats fed both 3'MeDAB-containing diets, for RNA in all treated groups (the smallest increase being in the group fed 3'MeDAB) and for protein in the group fed 3'MeDAB. Further analysis of the increases in total nuclear protein induced by pair feeding the experimental diets revealed that there were minor increases in nuclear sap proteins, histones and acidic nuclear proteins (Table 3). The increases were significant in the instances of nuclear sap proteins in rats fed both of the 3'MeDAB-containing diets, histones in rats fed the 3'MeDAB diet and acidic nuclear proteins in rats fed the diet containing both 3'MeDAB plus CAP.

The amount of protein in the crude enzyme extracts (fraction IV protein) was significantly increased after 4 days of pair feeding diets containing 3'MeDAB when results were expressed either as protein extracted/4 g liver (Fig. 3) or as protein extracted/liver/100 g body wt (Table 4). Estimation of total nuclear protein both before and after the extraction procedure showed that only 4–6% of the total nuclear proteins were extracted; this experiment also confirmed the increased extraction of protein from the nuclei of rats fed 3'MeDAB (results not shown). More detailed analysis of

Table 2. Effect of 4 days of pair-feeding a control diet and diets containing 2% CAP, 0·06% 3'MeDAB and 0·06% 3'MeDAB plus 2% CAP on the DNA, RNA and protein content of rat liver nuclei

Diet	DNA	RNA	Protein
Control	5·45 ± 0·04 (−)	1·03 ± 0·03 (−)	21·44 ± 0·87 (−)
CAP	6·37 ± 0·75 (+16·8)	1·29 ± 0·15 (+26·0)†	25·17 ± 2·24 (+17·4)
3'MeDAB	6·47 ± 0·48 (+18·7)†	1·15 ± 0·05 (+12·0)†	25·67 ± 1·15 (+19·8)*
3'MeDAB plus CAP	6·11 ± 0·35 (+12·0)†	1·24 ± 0·10 (+20·4)†	24·67 ± 2·18 (+15·1)

Each value is expressed as mg/liver/100 g body wt and represents the mean ± S.D. of data from 3 rats.
A total of 12 rats (mean wt ± S.D.; 140 ± 5 g) were used.
The figures in parentheses represent the percent difference from the respective control values.

*Significantly different from the control group ($P < 0·01$).
†Significantly different from the control group ($P < 0·05$).

Table 3. Effect of 4 days of pair-feeding a control diet and diets containing 2% CAP, 0·06% 3'MeDAB and 0·06% 3'MeDAB plus 2% CAP on the protein composition of rat liver nuclei before and after the extraction of fraction IV *protein*

Diet	Before extraction			After extraction		
	Nuclear sap protein	Histone	Acidic nuclear protein	Nuclear sap protein	Histone	Acidic nuclear protein
Control	5·52 ± 0·05 (−)	10·45 ± 0·71 (−)	3·84 ± 0·09 (−)	4·82 ± 0·10 (−)	9·84 ± 0·70 (−)	3·40 ± 0·34 (−)
CAP	6·45 ± 0·88 (+16·8)	12·83 ± 1·37 (+22·8)	4·59 ± 0·85 (+19·4)	5·68 ± 0·70 (+18·0)	12·30 ± 1·43 (+24·9)	4·02 ± 0·18 (+18·3)†
3'MeDAB	6·42 ± 0·48 (+16·2)†	12·61 ± 0·86 (+20·7)†	4·69 ± 0·66 (+22·0)	5·02 ± 0·28 (+4·2)	12·07 ± 0·90 (+22·6)†	3·95 ± 0·47 (+16·1)
3'MeDAB plus CAP	6·06 ± 0·32 (+9·8)†	12·02 ± 1·10 (+15·04)	4·41 ± 0·09 (+14·8)*	5·42 ± 0·31 (+12·6)†	11·69 ± 0·74 (+18·8)†	3·72 ± 0·67 (+9·49)

The results are expressed as mg protein/liver/100 g body wt and each value represents the mean ± S.D. of data derived from 3 rats. A total of 12 rats (mean wt ± S.D.; 140 ± 5 g) were used. Figures in parentheses represent the percent difference from the respective control values.

*Significantly different from the control group ($P < 0·01$).
†Significantly different from the control group ($P < 0·05$).

the protein content of nuclei that had been subjected to extraction suggested a selective extraction of nuclear sap proteins in rats fed 3'MeDAB and possibly acidic nuclear proteins in rats administered both 3'MeDAB plus CAP as compared with control rats (Table 3).

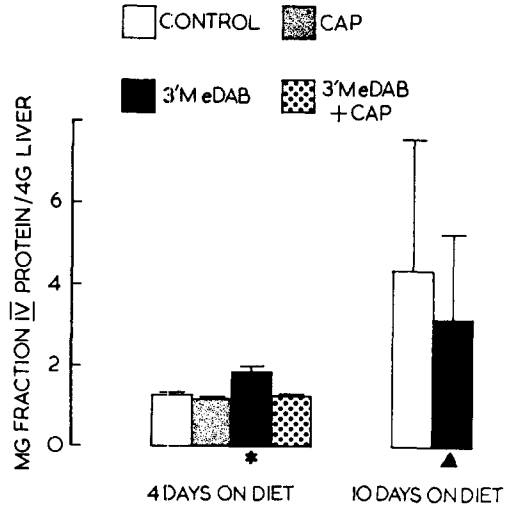

Fig. 3. Comparison of amounts of fraction IV protein extracted from the livers of rats which were pair-fed the various diets for 4 or 10 days. Each bar represents the mean ± S.D. of data derived from 3–8 rats; a total of 28 rats in all (mean wt ± S.D.; 147 ± 8 g) were used. The asterisk indicates a value that is significantly different from that of the control group ($P < 0·01$). The black triangle indicates a value that is significantly different from that of the control group only if the results are expressed as a percentage of the control value ($P < 0·01$. one-tailed t-test).

Characteristics of RNA synthesis catalyzed by fraction IV protein

The fraction IV protein samples were tested against standard rat liver DNA templates for their ability to catalyze RNA synthesis. In preliminary studies using a fraction IV protein preparation from a control rat, the amount of product formed increased with incubation time for 20 min. The reaction rate was determined by the amount of RNA polymerase present (DNA excess) when ≥ 15 μg of DNA and ≤ 250 μg of protein were used/assayed and under these conditions there was a linear increase in the amount of product formed when increasing amounts of fraction IV protein were added.

Rats pair-fed the diets containing CAP and 3'MeDAB plus CAP for 4 days showed a significant increase in the RNA polymerase specific activity (pmole ^{14}C-UTP incorporated/mg protein) of fraction IV protein preparations (Fig. 4). Although there was also a significant increase in the RNA polymerase activity of fraction IV protein preparations from rats fed 3'MeDAB alone for 4 days (Table 1), greater amounts of protein were extracted from the nuclei of these rats as compared with those receiving the other experimental diets (Table 4). Consequently, the specific activity of the RNA polymerase in the fraction IV protein from the rats fed 3'MeDAB for 4 days did not significantly differ from that of the control group (Fig. 4). However, if rats were fed the

Table 4. Effect of pair-feeding a control diet and diets containing 2% CAP, 0·06% 3'MeDAB and 0·06% 3'MeDAB plus 2% CAP to rats for either 4 or 10 days on the protein content of the nuclear extract

Diet	4 days on diet			10 days on diet		
	Fraction IV protein	RNA polymerase I-associated protein	RNA polymerase II-associated protein	Fraction IV protein	RNA polymerase I-associated protein	RNA polymerase II-associated protein
Control	1151 ± 101 (6)	11·53 ± 1·93 (5)	17·44 ± 2·80 (5)	3597 ± 2384 (3)	76·09 ± 42·92 (3)	72·99 ± 54·96 (3)
CAP	1169 ± 60 (3)	11·29 ± 1·31 (3)	13·93 ± 3.46 (3)	—	—	—
3'MeDAB	1775 ± 213* (8)	17·50 ± 6.70 (5)	21·30 ± 10·30 (5)	3673 ± 2515 (3)	34·00 ± 14.00‡ (3)	110·65 ± 78·53‡ (3)
3'MeDAB plus CAP	1337 ± 82*† (5)	13·07 ± 3·00 (3)	17·11 ± 2·12 (3)	—	—	—

Each value is expressed as μg protein/liver/100 g body wt and represents the mean ± S.D. of estimations performed on the number of animals shown in parentheses. These values have been corrected for losses occurring during chromatography by using the percent recovery data given in Table 7. A total of 28 rats (mean wt ± S.D.; 147 ± 8 g) were used in these experiments. The corresponding data for RNA polymerase activity are given in Table 1.

*Significantly different from the control group ($P < 0·01$).
†Significantly different from the 3'MeDAB group ($P < 0·01$).
‡Significantly different from the control group ($P < 0·025$, one-tailed Student's *t*-test) if the data is expressed as percent difference from control.

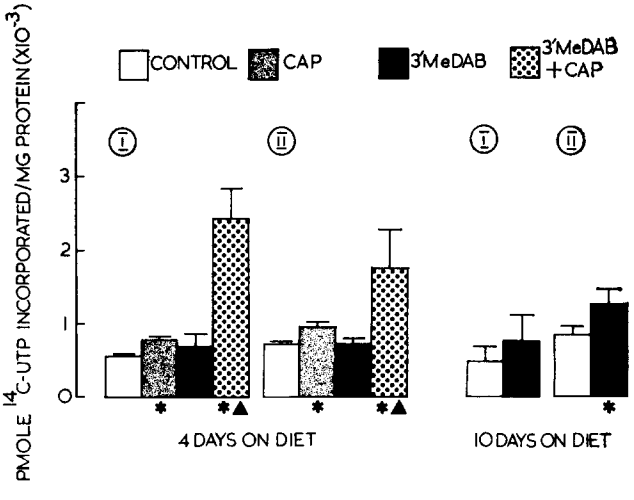

Fig. 4. Comparison of specific activity of RNA synthesis by fraction IV proteins isolated from the livers of rats that were pair-fed one of the various diets for either 4 or 10 days. The proteins were extracted from the liver nuclei by the method of Jacob et al. [8] *and aliquots (0·1 ml, ca 20–35 μg protein) were assayed against a standard rat liver DNA template (14·6 μg). The Mg^{2+}-stimulated and $Mn^{2+}/(NH_4)_2SO_4$-stimulated activities are shown beneath the symbols I and II respectively. Each bar represents the mean ± S.D. of data derived from 3–8 rats. A total of 28 rats (mean wt ± S.D.; 147 ± 8 g) were used in these experiments. The asterisks indicate values that are significantly different from those of the control group ($P < 0·01$). The black triangles indicate a significant difference between the group fed the 3'MeDAB-containing diet and that fed both 3'MeDAB plus CAP ($P < 0·01$). The values for the total RNA polymerase activity of these preparations are given in Table 1.*

3'MeDAB-containing diet for 10 days, there was a significant increase (relative to the control group) in the specific activity of the $Mn^{2+}/(NH_4)_2SO_4$-stimulated RNA polymerase activity, for greater amounts of enzyme activity were recovered from the nuclei of these 3'MeDAB-fed rats while the amount of protein extracted was similar to that in the respective control group (Tables 1 and 4). Differences in RNA polymerase activity in fraction IV protein preparations from rats fed the control and experimental diets were probably not due to an altered affinity of the enzymes for the substrate UTP. The K_m, determined by regression analysis [46], was virtually unchanged following any of the dietary regimens (Table 5).

Chromatography of fraction IV proteins on DEAE Sephadex A-25

When fraction IV protein from a control rat was applied to a column of DEAE Sephadex A-25, there was a large flow through peak of protein that eluted with the void volume. Two distinct peaks of RNA polymerase activity eluted at 0·14 M $(NH_4)_2SO_4$ and 0·26 M $(NH_4)_2SO_4$ respectively. Feeding the rats on the various diets had no significant effect on either the elution profile or the molarity of $(NH_4)_2SO_4$ at which the peaks of enzyme activity were eluted. The first and second

Table 5. Effect of pair-feeding a control diet and diets containing 2% CAP, 0·06% 3′MeDAB and 0·06% 3′MeDAB plus 2% CAP to rats for 4 days on kinetic parameters of RNA polymerase activity present in crude enzyme (fraction IV protein) preparations

Diet	Polymerase I			Polymerase II				
	V_{max} (pmole/mg protein)	% Difference from control	K_m ($\times 10^4$ M)	% Difference from control	V_{max} (pmole/mg protein)	% Difference from control	K_m ($\times 10^4$ M)	% Difference from control
Control	609 ± 8	—	0·969 ± 0·029	—	769 ± 11	—	1·071 ± 0·034	—
CAP	1195 ± 22	+96	0·934 ± 0·041	−3·6	1115 ± 19	+45	1·097 ± 0·041	+2·4
3′MeDAB	801 ± 7	+32	1·021 ± 0·019	+5·4	884 ± 12	+15	1·131 ± 0·033	+5·6
3′MeDAB plus CAP	2258 ± 49	+271	0·946 ± 0·047	−2·4	1477 ± 43	+92	1·029 ± 0·066	−3·9

Determinations were made using fraction IV protein preparations derived from 4 rats (mean wt ± S.D.; 165 ± 7 g); one rat in each dietary group. Incubations were performed as described by Jacob *et al.* [8]; Mg^{2+}-stimulated activity is designated as RNA polymerase I and $Mn^{2+}/(NH_4)_2SO_4$-stimulated activity as RNA polymerase II. Each assay tube contained 47·9 μg DNA and 3 μCi of ^{3}H-UTP and varying amounts of unlabelled UTP (0·05, 0·07, 0·09, 0·10, 0·11 and 0·13 μmole) were included in the incubation mixtures. At the conclusion of the incubations, the $HClO_4$ precipitates were processed according to the modification of the Blobel and Potter [23] procedure described in "Material and Methods". The data shown represent the means ± S.E. determined by regression analysis [46].

Table. 6. *Effect of α-amanitin on the incorporation of UTP into RNA by whole nuclei, fraction IV protein and purified RNA polymerases from normal rat liver*

Tissue fraction	Assay conditions	% Inhibition of UTP incorporation by α-amanitin
Nuclei	Mg^{2+}(pH 8·5), 6 min	22·8
	$Mn^{2+}/(NH_4)_2SO_4$(pH 7·5), 15 min	83·1
Fraction IV protein	Mg^{2+}(pH 8·0), 20 min	17·7
	$Mn^{2+}/(NH_4)_2SO_4$(pH 8·0), 20 min	86·4
DEAE Sephadex A-25 Peak I	Mg^{2+}/Mn^{2+}(pH 8·0), 20 min	8·0
DEAE Sephadex A-25 Peak II	Mg^{2+}/Mn^{2+}(pH 8·0), 20 min	96·5

α-Amanitin (0·08 μg in 50 μl deionized water) was added to the media just prior to initiation. The results shown are the mean of duplicate estimations. Nuclei were isolated by the method of Blobel and Potter [18] from the liver of a normal male Sprague–Dawley rat (156 g).

peaks of enzyme activity were equated with RNA polymerases I and II of Roeder and Rutter [6] (or A and B of Chambon *et al.* [47]) on the basis of their order of elution from DEAE Sephadex and relative sensitivities to the fungal toxin α-amanitin (Table 6). In agreement with others [48, 49], we found that RNA polymerase I activity was more labile than that of RNA polymerase II (Table 7). Accordingly, the total amounts of extractable RNA polymerase activity (Table 1) and the total amounts of fraction IV and enzyme-

Table 7. *Recovery of protein and RNA polymerase activity following DEAE Sephadex A-25 chromatography of fraction IV protein preparations from rats pair-fed a control diet and diets containing* 2% *CAP*, 0·06% *3′MeDAB and both* 0·06% *3′MeDAB plus* 2% *CAP for* 4 *or* 10 *days*

Diet	Protein recovered (% of original)*		RNA polymerase activity (% of original)*			
			4 Days on diet		10 Days on diet	
	4 Days on diet	10 Days on diet	Polymerase I	Polymerase II	Polymerase I	Polymerase II
Control	96·3 ± 6·1 (5)	98·2 ± 4·0 (3)	81·2 ± 10·1 (5)	96·4 ± 4·2 (5)	70·8 ± 16·0 (3)	89·1 ± 5·8 (3)
CAP	102·2 ± 5·7 (3)	—	83·1 ± 13·8 (3)	92·6 ± 7·1 (3)	—	—
3′MeDAB	97·5 ± 10·2 (5)	95·7 ± 2·7 (3)	82·5 ± 15·7 (5)	94·2 ± 13·3 (5)	66·2 ± 9·6 (3)	86·0 ± 13·5 (3)
3′MeDAB plus CAP	101·3 ± 9·6 (3)	—	72·2 ± 16·3 (3)	98·7 ± 9·3 (3)	—	—

Each value shown represents the mean ± S.D. of estimations made following DEAE Sephadex chromatography of fraction IV proteins from the number of individual rats shown in parentheses. In each instance, no statistically significant differences were noted between the values obtained from rats fed the various experimental diets and the rats fed the control diet. Polymerase I and II activities refer to the pooled activities of the first and second enzyme peaks that eluted from the columns. The corresponding data for RNA polymerase activity and protein content of these preparations are given in Tables 1 and 4 respectively.

*The original amounts of protein were those contained in the various fraction IV protein samples that were subjected to chromatography. The original amounts of RNA polymerase activity refer to the amounts of Mg^{2+}-stimulated activity (in the instance of polymerase I) and $Mn^{2+}/(NH_4)_2SO_4$-stimulated activity (in the instance of polymerase II) in the same samples.

associated protein (Table 4) have been corrected for any losses that occurred before or during chromatography.

Determination of the specific activity of RNA polymerases purified by DEAE Sephadex chromatography is subject to two major sources of error. The first is that of contamination of the enzyme peaks with other proteins that lack polymerase activity [50]. This source of error can be at least partly overcome by further purification procedures, but the lability of RNA polymerase I activity makes such attempts impracticable. The second problem, which has been stressed by Jacob [42], is to find a very sensitive method of protein analysis that is not affected by components of the elution buffer. The procedure described by Murphy and Kies [36] fulfilled these requirements and was routinely used for the protein analysis of the column effluents in the present study.

Both polymerases I and II, when partially purified by chromatography, exhibited a significantly increased specific activity in rats that were fed CAP or 3'MeDAB plus CAP for 4 days (Fig. 5). In particular, the specific

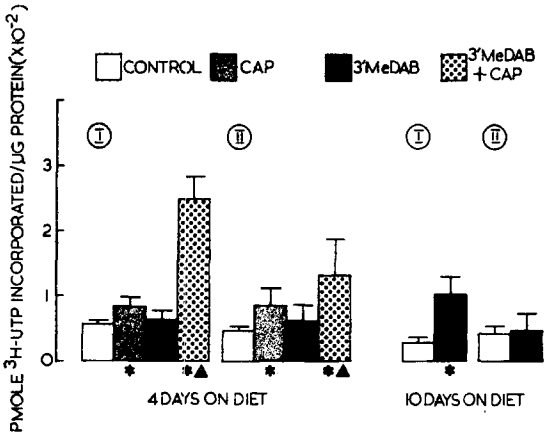

Fig. 5. Comparison of specific activity of RNA synthesis by soluble RNA polymerases I and II isolated from the livers of rats that were pair-fed one of the various diets for either 4 or 10 days. Samples of fraction IV *protein were chromatographed on DEAE Sephadex A-25 as described in 'Material and Methods' and fractions collected and assayed for RNA polymerase activity against a standard rat liver DNA template* (14·6 µg). *The values obtained for the respective peak fractions containing the enzyme activities were pooled. The symbols* I *and* II *refer to polymerases* I *and* II *respectively, the enzymes being distinguished by their order of elution and relative sensitivity to the toxin α-amanitin. Each bar represents the mean ± S.D. of data derived from* 3–5 *rats; a total of* 22 *rats (mean wt ± S.D.;* 147 ± 9 *g) were used. The asterisks indicate values that are significantly different from those of the control group* ($P < 0·05$). *The black triangles indicate a significant difference between the group fed the 3'MeDAB-containing diet and that fed both 3'MeDAB plus CAP* ($P < 0·05$). *The values for the total RNA polymerase activity of these preparations are given in Table* 1.

activity of these enzymes was much greater in rats fed the diet containing both 3'MeDAB and CAP (which protects against 3'MeDAB hepatocarcinogenesis) than in rats fed only 3'MeDAB. This effect was most pronounced in the case of RNA polymerase I activity. The specific activities of RNA polymerases I and II were not significantly different from the control values after 4 days of feeding only 3'MeDAB, but there was a significant increase in the case of RNA polymerase I if the diet was fed for 10 days. Changes in the specific activities of the RNA polymerases were mostly due to alterations in enzyme activities (Table 1) as opposed to alterations in the amounts of protein that chromatographed with each enzyme peak (Table 4). The proteins from the pooled peak fractions of polymerase activity were subjected to electrophoresis in polyacrylamide gels under non-denaturating conditions. Polymerases I and II both comprised two prominent bands of protein, a finding which is in agreement with earlier reports of their heterogeneity [50, 51]. The experimental diets did not significantly influence either the mobility or the staining intensity of the bands (results not shown).

The nature of the protein eluted in the flow-through peak was investigated. This peak comprised 69–83% of histone as determined by the method of Mirsky and Pollister [12] and these values are probably underestimated because of the limits of sensitivity of the reaction. A comparison of the histone contents of fraction IV protein samples from control and 3'MeDAB-fed rats revealed that they were almost identical (47% and 50% respectively).

DISCUSSION

The present study was undertaken with the aim of investigating the increase in nuclear RNA synthesis that takes place in the livers of rats that are protected against the hepatocarcinogenic azo dye 3'MeDAB by the antibiotic CAP. Nuclei from the livers of the protected rats showed early marked increases in both Mg^{2+}- and $Mn^{2+}/(NH_4)_2SO_4$-stimulated RNA synthetic activities (especially the former) when compared with those from rats fed the carcinogen alone for a similar period, in which the level of activity was little different from that of the controls [4]. The results presented in this report confirm those findings and suggest that the increases in nuclear RNA synthesis are at least in part the result of increases in soluble RNA polymerase activity, the most pronounced increase taking place in the activity of RNA polymerase I. Furthermore,

kinetic studies indicate that the increases in both RNA polymerase I and II activities are probably not due to an altered affinity for the substrate UTP. Caution is however necessary for the unequivocal interpretation of experiments where relatively crude polymerase preparations, such as those used in this study, are incubated with DNA. Several nuclease activities are present in similar preparations and have led to misinterpretation of the significance of altered levels of precursor incorporation into RNA catalyzed by those preparations [52]. Nicking enzymes, by creating artificial promoter sites in the DNA, increase incorporation [53], while other nucleases may degrade the reaction product [4] and/or inactivate the template [53].

Inhibition of azo dye-induced hepatocarcinogenesis has been shown to be accompanied, in the instances of the inhibitors CAP [4] and nitrofuran [54, 55], by an increase in Mg^{2+}-stimulated nuclear RNA synthesis. With the proviso mentioned above regarding possible nuclease contamination of the polymerase preparations, the findings of the present study suggest that with CAP, this increase is at least partly a consequence of increased polymerase I activity.

A rapid increase in the activity of hepatic RNA polymerase I has likewise been shown to follow partial hepatectomy [56–58] and the administration of certain hormones [59–62] and there is evidence suggesting that some of these alterations are due to allosteric changes rather than increased enzyme synthesis [59]. RNA polymerase molecules appear to have extended half-life periods and represent a fairly stable population [63], which is further evidence in support of the concept of modulation of RNA polymerase activity by allosteric change.

There are several reports in the literature concerning the effects of chemical carcinogens on soluble RNA polymerase activities in target organs. Thus the hepatocarcinogens aflatoxin B_1 [24], ethionine [64] and N-hydroxy AAF [65, 66] have all been shown to decrease the activity of either one or both of the soluble polymerases extractable from rat liver nuclei and the reports indicate that there is a tendency for polymerase II activity to be selectively decreased. Akinrimisi *et al.* [67] were able to show that the effects of aflatoxin administration *in vivo* on RNA polymerase II were mimicked by a metabolite of aflatoxin that was generated by an *in vitro* microsomal system. In contrast, Wu and Smuckler [68], who used the method of Cunningham *et al.* [69] to isolate the soluble nuclear polymerase, reported that the azo dyes, when given p.o., did not significantly affect RNA polymerase activity. Nevertheless, the synthetic ester N-benzoyloxy MAB, which is chemically related to postulated azo dye metabolites [70], was shown by them to inhibit the enzyme. The enzyme isolated by Cunningham *et al.* [69] was probably RNA polymerase II because of its stability and its salt optima. In all of these reports of the effects of carcinogens on polymerase activity, the carcinogens were administered in large single or repeated doses. Such experimental conditions are quite different from those routinely employed in tumour induction experiments and in the present study, where the carcinogen was incorporated in the diet at a low dosage level and fed continuously for weeks or months.

Continuous administration of a diet containing 0·06% 3′MeDAB was shown by us to have no significant effect on either RNA polymerase I or II activity if it was fed for only 4 days, a similar result to that obtained by Wu and Smuckler [68] when they administered azo dyes p.o. in acute doses. If a diet containing CAP was fed for 4 days, increases in the activity of soluble RNA polymerases I and II were observed, but these were significantly less than those in the rats fed both 3′MeDAB and CAP. The contribution made by CAP to the increase in polymerase activity therefore could not fully account for the increases noted in the protected rats after only 4 days on the diet. These findings led us to suspect that although RNA polymerase activity was not actually decreased by 3′MeDAB in our experiments, the increase in enzyme activity that might be expected to take place in response to cellular injury could be delayed in the livers of rats fed this carcinogen. If this is so, there should be an increase in RNA polymerase activity in the livers of rats fed 3′MeDAB for periods of longer than 4 days. In agreement with our hypothesis, when the diet containing the dye was fed for 10 days, an increase in both polymerase I and II activity was noted; this increase was less marked than that seen in the protected rats after only 4 days on the diet.

Total liver DNA increases to a similar extent in 3′MeDAB-fed rats and in rats protected from hepatocarcinogenesis by concurrent CAP administration [3], the decreased RNA/DNA ratio in rats fed 3′MeDAB [1, 3] being the result of a lag in production of liver RNA. The protected rats, in contrast, show no such lag, the liver RNA and DNA contents increasing in synchrony and the liver RNA/DNA ratio remaining at control levels [1, 3]. Previous

work indicates that alterations in RNA polymerase I activity are closely associated with alterations in ribosomal RNA synthesis [71]. The increase in RNA polymerase I activity in the livers of the protected rats is therefore probably largely responsible for the increase in liver RNA content and for restoring the RNA/DNA ratio to normality in these rats.

The enhanced activity of RNA polymerase I in the protected rats may ensure that ribosomes are available for the synthesis of the new protein that would be required following cellular damage. The situation would be somewhat analagous to the increase in polymerase I activity in liver that occurs prior to the mitotic response that follows partial hepatectomy [56–58]. The concomitant increase in the protected rats in the activity of RNA polymerase II, which is localized in the nucleoplasm and implicated in the synthesis of messenger-like RNA [6], might ensure that new m-RNAs are produced to direct the synthesis of these new proteins. The increases in RNA polymerase I and II activities in the protected rats could therefore be ultimately related to the normal mitotic response in these animals, for they might ensure, for example, that the spindle proteins would be coded for and produced at the appropriate time.

Some provisos, however, must be made regarding the interpretation of our findings. The first concerns the extrapolation of results obtained using extracts of nuclei to the intact liver. The extent to which the isolated nuclei represent the total nuclear population in the liver was discussed in our earlier report on the effects of 3'MeDAB and CAP on rat liver nuclear RNA synthesis [4]. A second problem is that only a few of the cells present in a tissue are at risk of transformation to tumour cells [72]. Furthermore, possible toxic effects of 3'MeDAB and CAP must be discriminated from pre-carcinogenic effects and effects necessary for the inhibition of carcinogenesis.

An increase in the amount of protein in the crude enzyme extract (fraction IV protein) obtained from liver nuclei after only 4 days of feeding the carcinogen was prevented by concurrent administration of CAP in the diet. As there were only minor effects of these diets on the total amount of nuclear protein and on the relative amounts of the major subclasses, the increase in extractable protein could be related to an early structural change within the nucleus induced by the carcinogen and prevented by CAP. There is evidence of nucleolar segregation and occasional nucleoli with condensations of the fibrillar component following 3'MeDAB administration [73] and these changes have been reported to be reversed by another inhibitor of carcinogenesis [74]. In addition, an increase in the nuclear sap proteins was noted in rats fed 3'MeDAB for 4 days. This increase, as well as the increased protein content of the crude enzyme extract, could be a consequence of 3'MeDAB-induced nuclear membrane alterations. Administration of the related hepatocarcinogen N-hydroxy AAF to rats is known to produce alterations in the nuclear membrane [75].

We did not find any direct evidence in these experiments for the presence of RNA polymerase III and there are several possible explanations of this observation. First, there are reports that polymerase III (or C) is located predominantly in the cytoplasm [76, 77] and our soluble RNA polymerase preparations were extracts of isolated liver nuclei. Furthermore, there is evidence that RNA polymerases I and III may be selectively lost during isolation of a crude nuclear pellet in isotonic buffer solutions [21]. In addition, RNA polymerase III is not adequately separated from RNA polymerase II by chromatography on DEAE Sephadex [76, 78] and has an intermediate sensitivity to α-amanitin [76–78]. Trace amounts of RNA polymerase III could therefore have cochromatographed with RNA polymerase II in the present study. Evidence that supports this notion is our finding that the second major peak of enzyme activity that elutes from the DEAE Sephadex column (which we have called RNA polymerase II) was not completely inhibited by α-amanitin.

Why is there little alteration in liver RNA polymerase activity (especially in RNA polymerase I activity) in the early stages of feeding the carcinogenic azo dye 3'MeDAB? When an increase finally takes place, it is concomitant with, rather than preceding the significant increase in liver DNA [3]. This sequence of events is at variance with that in the liver following partial hepatectomy [79] and suggests to us that some regulatory mechanism of RNA polymerase activity may be defective in the livers of rats fed the azo dye. RNA polymerases belong to the class of acid nuclear proteins [80], which have been strongly implicated in the regulation of gene activity [81]. It is therefore of some interest that the carcinogenic azo dyes DAB and 3'MeDAB have been shown to interact to a greater extent with acidic nuclear proteins than with histones [82, 83]. Whether 3'MeDAB or its metabolites interact

with the polymerases or with other protein regulatory factors and what such reactions, if demonstrated, would imply in terms of the regulation of cellular activities will require much further investigation.

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Correlation Between Urinary Steroids and Estrogen Receptor Content in Women with Early Breast Cancer*

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Abstract—*The urinary levels of* 11-*deoxy*-17-*ketosteroids and estrogen receptor content in tumor tissues were determined in* 76 *patients with early breast cancer. Twenty-six postmenopausal and* 16 *premenopausal patients with estrogen receptor negative tumors were found to excrete subnormal amounts of* 11-*deoxy*-17-*ketosteroids while* 22 *postmenopausal and* 12 *premenopausal patients with estrogen receptor containing tumors excrete near normal levels of urinary* 11-*deoxy*-17-*ketosteroids.*

INTRODUCTION

ENDOCRINE responsive tumors in women with metastatic breast cancer constitute only 20–40% of patients treated by surgical ablation or by pharmacologic hormone therapy. A number of clinical, pathologic, and biochemical criteria have been used in an attempt to predict the clinical course of patients with breast cancer after therapy [1].

Various studies have indicated a possible association between abnormal levels of androgen and corticosteroid metabolites in urine and plasma and the occurrence of breast cancer. The most convincing endocrine abnormality in patients with breast cancer has been found to be a subnormal excretion of 11-deoxy-17-ketosteroids (11-DOKS) [2–5] which can predict with considerable accuracy, either alone or in combination with urinary excretion of corticosteroids, the response to endocrine ablation therapy in patients with breast cancer. While these results have been supported by several investigators [4–7] others have disagreed with these findings [8–10]. The balance of evidence indicates that some steroid metabolites are present in abnormal concentrations in the urine and plasma of women, with breast cancer.

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Estrogen receptors (ER) have been demonstrated in approximately 70% of human breast cancer [11, 12]. The absence of ER almost always indicates that the tumor will be resistant to endocrine therapy while their presence is associated with a remission of advanced breast cancer in about 55% of cases [13].

In view of the fact that both an abnormality in urinary excretion of steroid metabolites and ER content in mammary tumors have been shown to be predictors of hormone responsiveness, the present study was undertaken to determine whether there is any correlation between the ER content in human breast cancer and the urinary excretion of 11-deoxy-17-ketosteroids.

MATERIAL AND METHODS

Control and cancer patients

The subjects were patients admitted to the American University of Beirut Hospital and came from various countries of the Middle East. Eighty-eight per cent of the patients were Lebanese. The rest came from Syria and Jordan. A total of 76 patients with primary breast cancer were included in this study (28 premenopausal, mean age 44·6 ± 4·4 years; and 46 postmenopausal, mean age 64·7 ± 6·3 years). The controls were 27 healthy premenopausal (mean age 43·3 ± 4·7 years) and 36 postmenopausal (mean age 62·4 ± 4·6 years) women, none of whom were taking any form of steroid preparations. The urine from control subjects was collected at home from normal

healthy women, but in 9 instances, the urine was obtained from patients in a geriatric ward.

Urine collection and analysis

Urine was collected from all patients 48 hr before operation. Urine analysis was done on 24-hr specimens stored at −20°C without preservative. The techniques used for the determination of 11-deoxy-17-ketosteroids (etiocholanolone + androsterone + dehydroepiandrosterone) were based on that described by Thomas *et al.* [14].

Estrogen receptor determination

Estrogen receptor content of the tumor samples was carried out using the dextran-charcoal coated technique as determined previously [15].

Expression and evaluation of results

The levels of urinary 11-deoxy-17-ketosteroids were calculated and expressed as means ± S.D. in mg/24 hr urine. Results were evaluated statistically by use of the *t*-test. Differences between means were considered statistically significant at $P < 0{\cdot}05$.

RESULTS

The urinary excretion of 11-DOKS has been estimated in normal women and patients with primary breast cancer. The mean excretion of 11-DOKS (Fig. 1) in the normal postmenopausal group was found to be 1·66 ± 0·88 mg/24 hr, 1·44 ± 0·54 mg/24 hr in the postmenopausal ER positive group and 0·75 ± 0·28 mg/24 hr in the postmenopausal ER negative group. There was no significant difference between the control group and the ER positive groups, $P < 0{\cdot}30$, while the difference between the control group and the ER negative group was found to be very significant, $P < 0{\cdot}001$.

Figure 2 shows the distribution and the means of urinary 11-DOKS in normal premenopausal women and patients with ER positive and ER negative primary breast cancer. The mean excretion was found to be 4·18 ± 0·47, 3·91 ± 0·42, and 3·66 ± 0·55 mg/24 hr, for the normal group, the ER positive group and the ER negative group, respectively. The results show that there was no significant difference between the control group and the ER positive group ($P < 0{\cdot}20$), but that there was a significant difference between the control group and the ER negative group ($P < 0{\cdot}005$).

The results show also that there is a significant difference between the mean levels of 11-DOKS in the pre- and postmenopausal control and cancer patients, there being a substantial reduction in 11-DOKS excretion after the menopause.

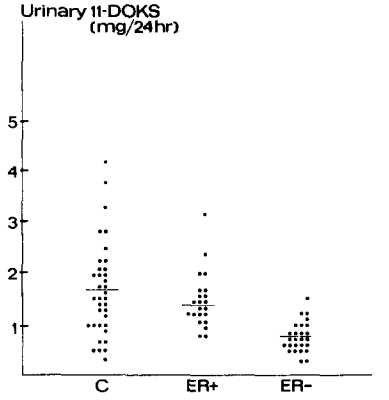

Fig. 1. Urinary 11-deoxy-17-ketosteroids (11-DOKS) by postmenopausal women in control subjects (C) and breast cancer patients with estrogen receptor positive (ER+) and estrogen receptor negative (ER−) tumors. The horizontal bars show the means.

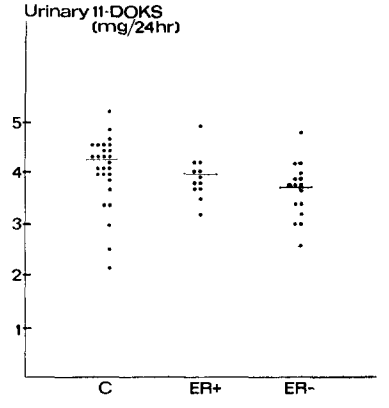

Fig. 2. Urinary 11-deoxy-17-ketosteroids (11-DOKS) by premenopausal women in control subjects (C) and breast cancer patients with estrogen receptor positive (ER+) and estrogen receptor negative (ER−) tumors. The horizontal bars show the means.

DISCUSSION

Abnormalities in urinary excretion of steroid metabolites have been observed by Bulbrook and his colleagues in women with both primary [3] and advanced [4] cancer of the breast and have been related to the prognosis and outcome of endocrine surgery. These observations were supported by several groups of investigators [6, 7] while others have presented evidence [8–10] in variance with that presented by

Bulbrook and his coworkers. However, it is generally accepted that significantly low levels of androgen metabolites are associated with poor prognosis in patients with advanced breast cancer.

The initial results of Jensen [16] on the use of ER content in breast tumor tissues for predicting response to endocrine therapy has been confirmed by other investigators [13]. The presence of ER in tumors of patients with advanced breast cancer increases the likelihood of response to endocrine therapy from about 25 to 55–60%. A favorable prediction based on ER alone will, therefore, prove incorrect in approximately 40% of cases. But, in ER negative tumors the chances of regression in response to endocrine therapy are minimal, and so selection based on the ER assay alone will still save many patients from profitless major endocrine surgery.

If in fact the low levels of urinary 11-DOKS excretion and the absence of ER are both predictors of hormone unresponsiveness, then they should correlate well in the same group of patients. On the other hand, cases in which hormone dependence has not been lost, one might expect to have a near normal level of urinary 11-DOKS as well as the presence of ER in the same group of patients. The results obtained from this study show that there is good correlation between the absence of ER and low levels of 11-DOKS in both pre- and postmenopausal patients, though the significance levels are different. Also, there was a good correlation between the presence of ER and the near normal values for 11-DOKS in both the pre- and postmenopausal group. There was no significant difference between the means for 11-DOKS in patients with ER positive tumors and the control groups, yet one observes a slight decrease in the means for 11-DOKS in both the pre- and postmenopausal group with mean differences of 0·27 and 0·22 mg/24 hr, respectively. This slight decrease in the mean level of urinary 11-DOKS in patients with ER positive tumors might be due to the contribution of the lower urinary 11-DOKS in those patients that do not respond to endocrine therapy, even though ER are present in the tumors of these patients.

Whilst it is now well established that absence of ER in a breast tumor almost invariably indicates that the tumor will be resistant to endocrine therapy [13], the situation regarding the urinary levels of androgen metabolites remains equivocal. However, the fact that a good correlation was obtained between the absence of ER, and the low levels of 11-deoxy-17-ketosteroids, suggests that patients unresponsive to endocrine therapy excrete subnormal amounts of 11-deoxy-17-ketosteroids. Furthermore, our results show that responsive patients, as determined by the presence of ER, excrete near normal values of 11-deoxy-17-ketosteroids even though about 40% of patients with ER positive tumors are endocrine resistant. The findings obtained from this study may imply that only those tumors growing in a normal environment respond to the alterations in the environment brought about by the treatment and it remains to be seen whether patients with ER positive tumors that do not respond to endocrine therapy have in fact a significantly lower level of urinary 11-deoxy-17-ketosteroids than patients with ER positive tumors that do respond to endocrine therapy.

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Radiofrequency Heating of Tumours in Rodents*

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Abstract—*This paper describes the results of treating* 170 *tumours in rodents at temperatures up to* 50°C *by radiofrequency (RF) heating. The apparatus used operates at a crystal-controlled frequency of* 13·56 *MHz* (22 *m wavelength*) *and the tumour is included in the output circuit by capacitive coupling between the electrodes. The paddle electrodes are applied directly to opposite sides of the tumour mass. The circuitry of the equipment incorporates special stabilising features which enable homogeneous tumour heating to be achieved easily and controllably.*

Two syngeneic tumours in the rat (D23 carcinoma amd MC7 sarcoma), and an allogeneic rat tumour (Yoshida sarcoma) growing subcutaneously in the foot or flank were heated at 45°C *intratumour temperature for* 15 *min. Ehrlich ascites tumours in mice were heated at* 42°C/1 *hr*, 43°C/30 *min or* 44°C/15 *min.*

Skin temperature over the rat tumours remained at 4–5°C *below that in the tumours. With the MC7 and Yoshida tumours treated at a volume of* 1–1·5 *ml, a* 100% *regression rate was obtained. With large* (6–12 *ml*) *Yoshida tumours in the leg muscles heated at* 45°C *for* 30 *min or* 50°C *for* 15 *min, all rats died soon after treatment. The D23 carcinoma* (0·7–1·5 *ml*) *was not cured by hyperthermia up to* 45°C *for* 15 *min. All tumour-bearing mice, and also a series of normal mice, died during or immediately after heating.*

When the normal leg of the rat was heated in the same RF field as the intramuscular Yoshida tumour, the tumour maintained a temperature 2–3°C *below that in the muscle. The electrical resistance of the heated tissue was not altered by the presence of the tumour. The results suggest that in the case of this tumour blood flow is greater than in adjacent normal tissue. This therefore places an upper limit on the temperature/time regimen that can be used to destroy the tumour.*

INTRODUCTION

THERE is growing interest in the use of hyperthermia (42°C and above) for the treatment of cancer. The approach exploits a fundamental difference in the thermal sensitivity of normal and malignant cells. There is evidence for this, both *in vitro*, where the metabolism of several types of cancer cell is irreversibly inhibited at 42°C [1–3], and *in vivo* where the selective heat destruction of a wide spectrum of tumours in animals [2, 4–8] and in man [1, 9–10] has been demonstrated.

There is also evidence of a potentiating effect of hyperthermia in the destruction of malignant cells by radiotherapy [11–13], chemotherapy [14–16] and cell specific antiserum [17].

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The destruction of tumour cells by hyperthermia is a complex process not yet fully understood. Response to heat in animals is governed by tumour volume, temperature and the duration of heating [18]. Cytokinetic studies *in vitro* and *in vivo* have shown a slow death of heated cell populations, involving competing processes of cell loss and repopulation [19, 20]. At the biochemical level, hyperthermic cell killing appears to be at least a two-stage process requiring the conversion of sublethal to lethal damage [14, 19]. Host factors also appear to be involved, as indicated by the regression of metastases in animals [2, 8] and in man [1] following curative local hyperthermia, increased tumour dissemination after inadequate tumour heating [18, 21] and apparent immunosuppression caused by total body hyperthermia in mice [22] and in rabbits [23].

Using presently available heating techniques for the treatment of cancer in humans, thera-

pists have been largely restricted to a temperature range of 41–43°C by considerations of host tolerance [1, 9, 24]. The general findings of this work have been initial regression of tumours followed by recurrence. In addition, human tumours *in vitro* have been shown to be less sensitive than animal tumours to 42°C [3]. The use of temperatures above 42°C offers a possible solution to this problem, although such temperatures involve the loss of the differential sensitivity between normal and malignant tissue, and the effectiveness of any such technique will depend on the degree to which adjacent normal tissues can be protected from heat damage. This report describes the destruction of rodent tumours by single short exposure to a low power radiofrequency field. The specially-designed radiofrequency machine enabled intra-tumour temperatures of up to 50°C to be achieved rapidly and controllably without damage to surrounding tissue.

MATERIAL AND METHODS

Tumours

Three chemically induced transplantable tumours of the rat were studied. The Yoshida tumour had a multifocal origin in the genital omentum, pelvic peritoneum and liver, following administration of O-aminoazotoluol to an albino rat and painting of the skin with potassium arsenite. It is classed as an undifferentiated tumour [25]. The tumour was maintained by periodic transfer of 0·1 ml (100 mg) tumour homogenate by trocar into the hind leg muscles of outbred Wistar rats. The D23 tumour is a syngeneic hepatocellular carcinoma that was produced by feeding young male rats a low protein diet containing 4-dimethylaminoazobenzene [26]. Sarcoma MC7 was the result of s.c. injection of 3 methylcholanthrene in trioctanoin into inbred female Wistar rats [27]. The D23 and MC7 tumours were passaged by serial transfer of 0·1 ml (100 mg) tumour homogenate s.c. into the flank of inbred Wistar rats.

For the present work, the Yoshida and MC7 tumours (100 mg) were transplanted into the dorsum of one hind foot of the rats. The Yoshida tumour was also treated at the implantation site in the leg muscles, and the D23 and MC7 tumours after s.c. transplantation into the flank, the initial inoculum in each case being 100 mg. At the time of implantation all rats weighed 200–250 g. From these various sites the untreated tumours metastasized to kill the host within 60 days.

The Ehrlich ascites tumour was maintained by serial passage of 10^7 cells in Tuck outbred (TO) adult white mice. Following this i.p. inoculation, the mice died in 10–14 days.

Tumour volumes were calculated from caliper measurements made in the antero-posterior, lateral and vertical planes. Allowance was made for the host's normal tissues on the basis of measurements made on each animal before inoculation of tumour.

Tumour heating

The apparatus for heating tumours by radiofrequency (RF) currents is shown in Figs. 1 and 2, and is available from Critical Systems Inc., Palo Alto, California. It consists of tuned oscillator and resonator circuits with refinements developed as a result of experiments using phantoms of isotonic NaCl gel as heating models. The generator has an output of 320 W at a crystal-controlled frequency of 13·56 MHz

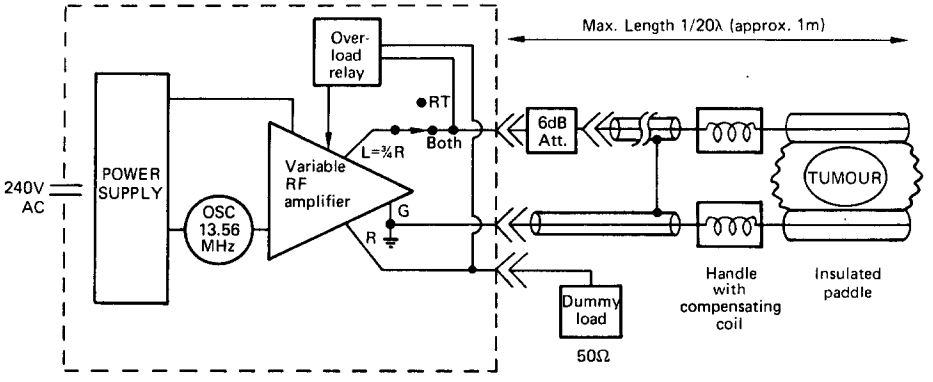

Fig. 1. Block diagram of RF heating system electronics. The broken square indicates the basic oscillator and output circuits which are contained in the portable RF machine (A) shown in Fig. 2. The tumour constitutes part of the dielectric of the capacitor formed by the heating paddles, and is coupled to the machine via two coaxial cables. The option switch shows both outputs of the machine driven, the L channel heating the tumour via the 6dB attenuator, and the R channel being directed into a 50Ω dummy load.

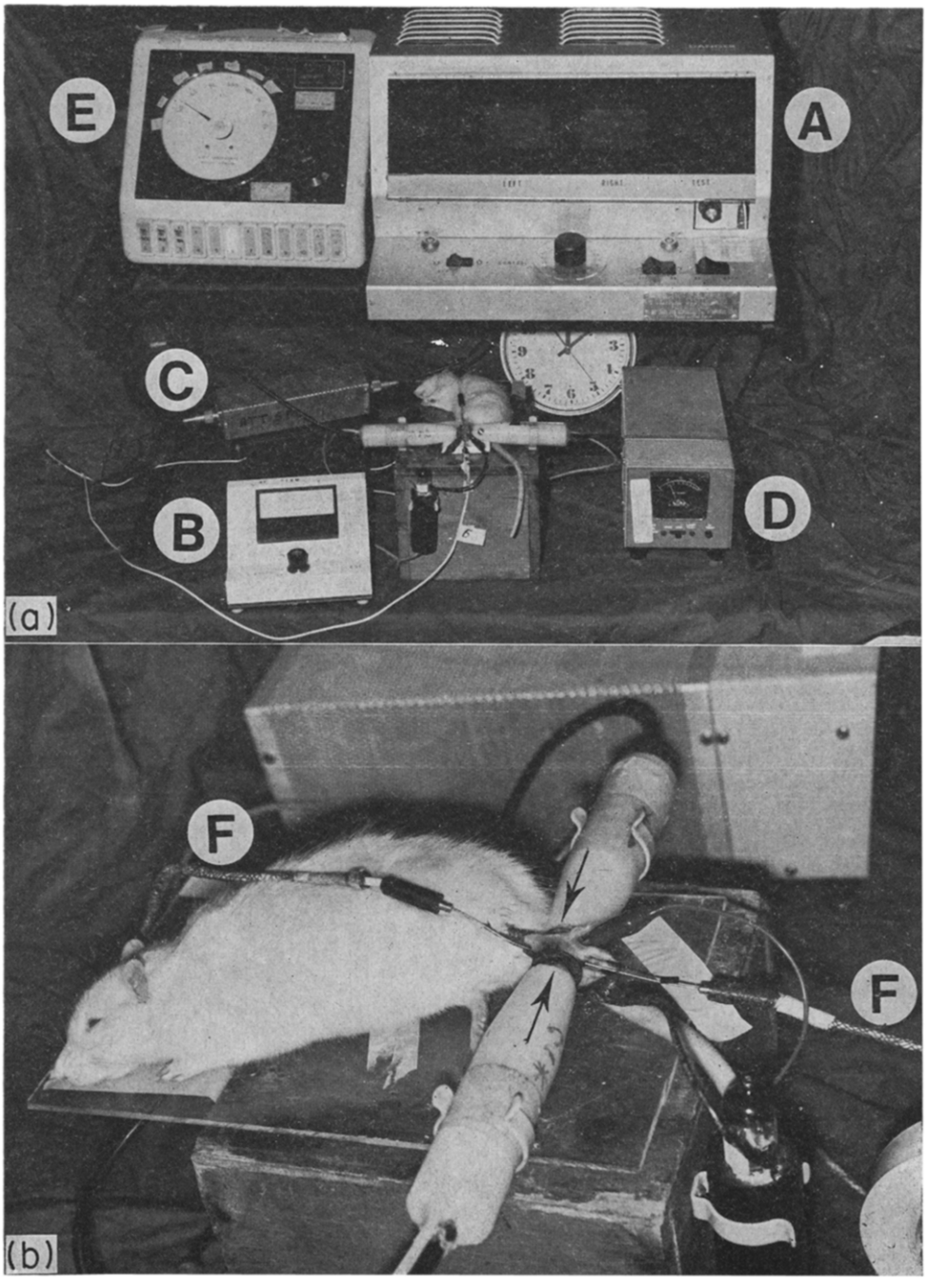

Fig. 2. Experimental arrangement for RF heating of rat foot tumour (a), and close-up of electrode configuration (b).

The high frequency current generated by the RF machine (A), and measured as amperes, is applied to the tumour via two heating paddles (arrows) with insulating handles. Potential drop across the tumour is measured by a valve voltmeter (B) attached to the electrodes. Low currents are obtained by inserting a resistance (attenuator, C) between the RF machine and the live paddle. The machine output channel not in use is fed into the 50 Ω dummy load (D).

Temperatures in the tumour and host are continuously monitored by direct readout on the 12 channel electric thermometer (E), coupled to multiple sensors (F) inserted at right angles to the RF field.

The RF current is transmitted from the amplifier to the tumour via an impedance matching circuit and two coaxial cables that terminate in flat paddle electrodes. When these electrodes are placed on diametrically opposite sides of the tumour mass, a condenser or electrostatic field is achieved, and the tissue becomes part of the dielectric of a capacitor. The paddles may be of various shapes and sizes depending on the dimensions of the tumour being heated, and are coated with a thin film of low-loss dielectric. This minimizes heating of the paddles, and in addition provides a high capacitive reactance minimizing the possibility of electric shock from low frequency switching transients. Tuned compensating inductance coils are contained in the insulating paddle handles to neutralize the capacitive reactance between the electrodes and the tissue, and thus maximize the heating component of the current in the tissue. An additional improvement in coupling the tissue to the electrodes was achieved by connecting the outer sheaths of the coaxial paddle cables through a "jumper" lead. This provides for optimum current flow between the heating paddles, and ensures that the ends of the cable sheaths are grounded with respect to RF voltage. The length of the coaxial cables from the generator to the electrodes was kept well below 1/10 of a wavelength (22 m), taking into account the velocity of propagation in coaxial cable (typically 60–70% of that in free space). This avoids the necessity of adding adjustable tuning controls to the equipment, and provides for accurate RF current measurements using panel meters.

The generator has a 2 channel output. When the L channel is used, the R channel is fed into a 50 Ω dummy load. Low currents are obtained by incorporating a 6dB attenuator into the L channel output. RF power is measured in amperes, and the potential drop across the tissue (V) by a high impedance vacuum tube voltmeter across the paddles. To provide high stability of the power control and to ensure a constant current for variations in spacing between electrodes, a d.c. inverse feedback loop was developed to inter-connect the output RF and the master crystal oscillator. An overload relay automatically shuts off the generator output at an RF current of 2·2 A, providing a necessary safety factor.

Temperature measurement

Temperatures were monitored by thermistor probes and a 12 channel direct reading electric thermometer (Model 3GID, Light Laboratories, Brighton, England) with a scale range of 36–56°C and an accuracy of ± 0·1°C. For intra-tumour, intra-abdominal or subcutaneous temperature measurement, the probes used were 3 or 5 cm long needle type IH 0·8 mm in dia, recording temperature change only at the needle tip; polythene-covered type IMR sensors were used for rectal recordings. The probes were individually earthed to the light meter and the cables were shielded to minimize interference from the RF field. The thermometers were standardized against a mercury-in-glass thermometer of the British Standards Institution, and the probes and meters were checked for "drift" before each days experiments.

At the low power outputs (< 6 W) used in the present work, the thermistor probes were unaffected by the RF field. That the sensors gave a valid measure of tumour temperature was confirmed by inserting thermistors into tumours immediately after the RF field was switched off. Tissue temperatures within the field were also checked by means of needle-type copper–constantan thermocouples.

Heating procedure

Anaesthesia was induced in the rats and mice with i.p. Sagatal, 0·1 ml of a 1:5 dilution per 50 g body weight (Sagatal Veterinary, 60 mg pentobarbitone sodium per ml: May and Baker) and maintained by additional small doses of the barbiturate as required.

All experiments were performed in a temperature-controlled room (25°C) and, where necessary, an i.r. lamp was used to minimize the initial fall in body temperature of the anaesthetized animals while they were prepared and the equipment positioned.

The animals were shaved with electric clippers or razor and final traces of hair were removed by a 5-min application of hair remover cream. The probes were then inserted. The tumour needles were placed in the proximal and distal poles, or centre of the mass, and the intra-abdominal sensor was introduced under the liver in a right paramedian position. Skin temperature was measured by a needle introduced s.c. between the tumour and heating paddle. All probes were positioned at right-angles to the RF field ensuring that there was no contact between paddle and needle. One surface of each paddle was coated lightly with conducting electrode jelly (Cam Creme, Kent Cambridge Medical Ltd.) and the two paddles applied to diametrically opposite sides of the tumour. The tumour was heated as part of a cylinder of tissue, the cylinder ends being the paddles, the paddle size selected and the cylin-

der volume heated being governed by the tumour size. Limb tumours and flank tumours were treated with the animal lying on its side (paddles applied to the medial and lateral aspects of the tumour); for the foot tumours, heat was applied by paddles held against the dorsum of the foot and the footpad. Flank tumours were also heated by "coning". A small heating paddle covered the tumour and formed the apex of the RF cone, while a larger ground paddle was applied to the opposite side of the animal abdomen constituting the base of the RF cone. Ascitic tumours in mice were treated by the cylinder technique, circular paddles being applied to opposite sides of the abdomen with the mouse lying on its back.

RESULTS

Figure 3 illustrates the temperature profiles and required RF power when Yoshida foot tumours of 1·0–1·5 ml vol were heated at 45°C

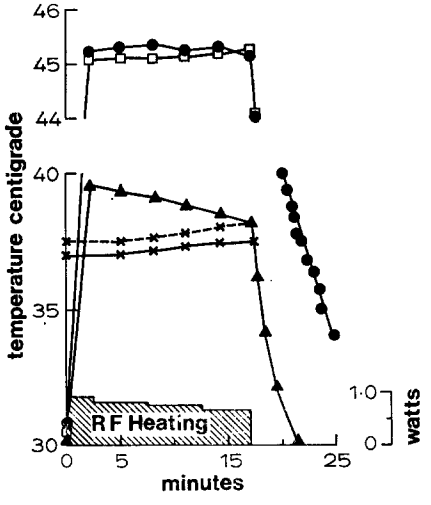

Fig. 3. Simultaneously recorded intra-tumour (● proximal pole of mass, □ distal pole), skin over tumour (▲), intra-abdominal (× – – – ×), and rectal (×——×) temperatures for a rat with a Yoshida tumour implanted on the dorsum of the left hind foot. The 1·2 ml tumour was treated by RF heating (intra-tumour temperature 45°C) for 15 min. Heating paddles were 1·5 cm in dia with a separation distance of 1·2 cm.

for 15 min. With an applied power of less than one Watt, the intra-tumour temperature increased rapidly to 45°C within 2–3 min. Homogeneous heating was maintained easily and controllably with a very low applied current (0·15–0·25 A). Skin temperature over the tumour did not exceed 40°C, and abdominal and rectal temperatures did not increase significantly. A cure rate of 100% has been obtained with 34 foot tumours 0·8–1·5 ml in volume treated at 45°C for 15 min. Eighteen Yoshida foot tumours of similar volume heated at 42°C for 1 hr were also cured. Tumour regression took place within 14 (45°C treated) or 22 (42°C treated) days. Four rats with bilateral 1·0–1·5 ml tumours were given RF treatment to one foot only (45°C for 15 min.) Both tumours regressed within 6 weeks in these animals.

MC7 sarcomas (1·0–1·5 ml vol) situated on the dorsum of the foot or on the flank were treated by RF cylinder heating using paddles applied above and below the foot, or paddles applied to each side of the tumour. A regression rate of 100% was achieved in both situations (12 foot, 16 flank tumours) following a 15 min heating session at 45°C intra-tumour temperature. Flank tumours of this volume were also cured by "coning" using a 2·5 cm dia paddle over the tumour and a 4·5 cm dia paddle applied to the opposite side of the abdomen. With this geometric cone, the animals intra-abdominal temperature remained below 40°C. When the 4·5 cm abdominal paddle was replaced by a 2·5 cm dia paddle, and the subcutaneous tumour heated at 45°C as part of an abdominal cylinder, all rats died soon after treatment. The temperature of the abdomen within the RF cylinder was 44–45°C.

A series of experiments were performed to investigate the pattern of heating of tumour and normal tissues exposed to the same RF field. The results are illustrated in Fig. 4.

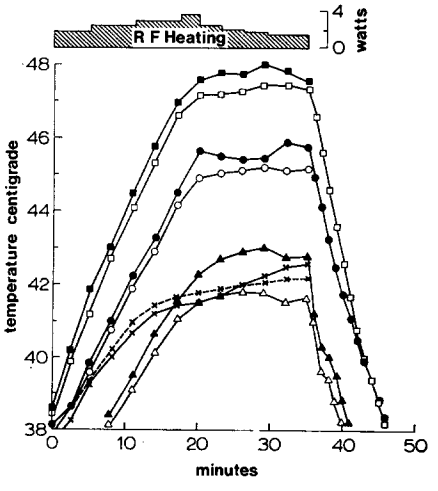

Fig. 4. Temperature profiles and cooling curves for RF heating of the two rear legs of a rat, one leg bearing a 6 ml intramuscular Yoshida tumour. A tissue cylinder was formed between the heating paddles by strapping the limbs together loosely at the ankles. Three cm diameter paddles were used at a separation distance of 2·7 cm. Tumour temperature (●, ○) was maintained at 45°C for 15 min. Concomitant temperatures achieved in normal muscle of the opposite leg (■, □), skin over the tumour (△), and skin over the normal muscle (▲), as well as rectal (× – – – ×) and intra-abdominal (×——×) temperatures are indicated.

Intramuscular Yoshida tumours of 5–10 ml in the rat were used. The tumour-bearing limb and the animal's normal limb were lightly strapped together at the ankle. A cylinder of tissue of diameter adequate to cover the tumour was heated, with one paddle applied to the tumour and the other to the lateral side of the normal leg. Passage of the current between the legs was facilitated by coating the apposed skin surfaces in the field with conducting jelly.

When the Yoshida tumour was heated at 45°C, the normal muscle of the rat maintained a temperature that was consistently 2–3°C higher than the tumour (Fig. 4). The heating and cooling curves for tumour and muscle were similar. There was little difference in skin temperatures under the paddles, both remaining below 43°C. Rectal and intra-abdominal temperatures increased progressively, and were in excess of 42°C during the latter part of the 15 min heating period. Seven rats were treated by this procedure. All animals survived the heating, but died within a further 48 hr.

Table 1 records the detailed electrical measurements obtained with RF heating of normal legs and tumour-bearing limbs in experiments similar to that depicted in Fig. 4. The tissues were brought to 45°C and maintained at this temperature for 15 min. From the mean amperage (I) and mean potential difference (V) across the tissue cylinder during the 15 min heating period, the tissue resistance (V/I) was calculated. The geometry of the heated tissues is indicated by the diameter and separation of the paddles, and the range of amperes and volts required to maintain these cylinders of different volume at temperature is given. The presence of tumour in the muscle did not significantly alter the electrical resistance of the leg(s). The resistance of rat leg muscle at 48°C (the temperature achieved in Fig. 4) was similar to the resistance at 45°C.

Unsuccessful treatment

Large Yoshida tumours (6–12 ml vol) situated in the leg muscles were treated at 45°C for 30 min (17 rats) or 50°C for 15 min (16 rats). To maintain these temperatures, the applied RF power did not exceed 5 W (10 V at 0·5 A) and usually less than 3·5 W sufficed. Rectal temperatures reached 41°C in 4 of the 45°C treated rats, but remained below 40°C in the others and in the 50°C treated animals throughout the heating; skin temperature over the tumours was consistently 4–6°C below intra-tumour temperature. All rats died within 72 hr of heating. At autopsy, no macroscopic lesion was observed that might have led to death of the host, and the tumour showed no gross lysis.

D23 tumours (0·7–1·5 ml vol) on the flank of rats were subjected to RF treatment regimens of 42°C/1 hr, 43°C/30 min, or 45°C/15 min. Cylinder heating by paddles applied to opposite sides of the tumour was employed. No regression occurred in a series of 20 tumours treated.

A series of mice bearing Ehrlich ascites tumour were treated 7 days after intraperitoneal inoculation of 10^7 cells. RF heating was by a cylinder using 2·0 or 2·5 cm paddles applied to opposite sides of the abdomen. An intra-abdominal temperature of 42, 43 or 44°C was maintained for 60, 30 or 15 min respectively (6 mice at each temperature), the applied power being less than 2 W. All mice died during or immediately after heating. At post-mortem there was acute haemorrhagic necrosis of the small intestine. Normal mice treated by RF (intra-abdominal temperature 42°C/60 min, 43°C/30 min, 44°C/15 minutes, 6

Table 1. *Protocol for maintaining normal and tumour-bearing limbs of the rat at* 45°C *by RF heating for* 15 *min*

	Volts (V)	Amps (I)	Resistance $\left(\Omega = \frac{V}{I}\right)$	Paddle diameter (cm)	Paddle separation (cm)
One leg (4)	3–6	0·1 –0·25	25·4 ± 4·0	1·5	1·0–1·3
Both legs (4)	6·4–10·1	0·2 –0·35	28·0 ± 3·9	2·0 or 2·5	2·0–2·7
One leg with tumour (6)	8–13	0·35–0·50	26·3 ± 4·7	2·5 or 3·0	2·5–3·2
Both legs, one with tumour (7)	5·2–10·5	0·2 –0·42	26·7 ± 2·0	3·0 or 3·5	2·7–3·8

The tissue was heated as a cylinder between two paddles of equal diameter applied to opposite sides of the leg(s). Paddle diameter was chosen to be greater than or equal to the thickness of the limb(s); with tumour-bearing legs, the paddle diameter was always greater than the tumour diameter measured in the same plane. The tumours ranged in volume from 4 to 10 ml. Resistance values are given ± S.D., and the figures in brackets represent the number of different animals examined in each group.

animals at each temperature) also died immediately after treatment. Acute necrosis of the small intestine was also present in these mice at autopsy.

DISCUSSION

Electrical approaches to the local heating of animal tumours are not new. Diathermy techniques have occupied most attention, and over the years wavelengths ranging from 500 m [28] to microwaves at 10 cm [29], have been employed. Most workers have been unable to obtain high percentage cure rates. In recent years, workers have concentrated on achieving intra-tumour temperatures of 42–44°C [6, 30], since at higher temperatures damage to normal tissues becomes a limiting factor.

The older literature contains reports on the electrical heating of animal tumours at temperatures in the region of 50°C. Overgaard and Okkels [28] treated Crocker sarcomas (approx. 0·1 ml vol) on the tail of mice with radiofrequency currents of wavelength 500, 20–30 or 5–7 m. Temperatures of up to 46°C were maintained for 15–60 min. A cure rate of less than 20% was obtained in a large series of tumours. Westermark [31] treated Flexner–Jobling and Jensen tumours growing s.c. in the flanks of rats. He used electrodes of unequal size (15–20 cm^2 probe applied to the tumour, and a ground electrode of 100 cm^2 on the opposite side of the animal body) and a current of 0·1–0·4 A, but unspecified frequency. Westermark obtained superficial to deep skin damage at tumour temperatures over 46°C. Johnson [32] gripped Walker 256 and Jensen tumours between two electrodes, and subjected them to short radio waves (λ3·4 m) at temperatures of 47°C for up to 45 min, or 50°C for 4 min. Damage to healthy skin occurred at temperatures in excess of 47°C. As found by Westermark, a considerable number of tumours treated at each temperature were unrestrained or regressed only temporarily after treatment. Cater *et al.* [29] achieved 45–47°C for 10 min in intramuscular hepatoma 223 tumours in the thigh of rats, using microwave heating at 10 cm wavelength. No tumour cures were obtained, and at 47°C oedema of the foot and areas of thrombosis in the tumour and overlying tissue occurred. Heating periods of over 10 min at 47°C incurred the danger of limb destruction.

The theory and use of radiofrequency currents in the short wave diathermy range (λ30–3 m) have been reviewed in detail [33–35], and the problems associated with obtaining selective heating of tumours by this means have been discussed by previous workers [6, 31, 32]. It has been repeatedly demonstrated that the depth of penetration of the heating effect, and the temperature distribution within the tissue, is critically dependent upon the relationship between the heated part and the electrodes, and on the geometry of the electrodes, as well as the current frequency employed [33, 34, 36]. In the present work, efficient contact between the paddles and skin was crucial. All traces of hair must be removed from the skin, thus decreasing greatly the high electrical resistance of this barrier. The distance between the paddles should not greatly exceed the paddle diameter, since the power compensation for varying plate separations takes place over a range of ± 20% approximately, where 100% represent plate diameter. Other important contributory factors in the readily-controlled and uniform tumour heating may be: (1) the use of the longest wavelength (22 m) approved by the Federal Communications Commission for medical use, with its greater depth of penetration in tissue and less heat generation in fat than shorter wave lengths [34, 37]; (2) employment of the electrostatic (condenser) field technique whereby the tissue becomes part of the resonator circuit, instead of the more commonly used inductive (electromagnetic) field method in which the tissues are not in the circuit but within the magnetic field of a coil; (3) the incorporation of tuned compensating coils in the paddle handles; and (4) the stabilising influence of the d.c. inverse feedback loop in the circuit.

LeVeen *et al.* [38] have recently reported results obtained from heating tumours in animals and humans with an RF apparatus operating at 13·56 MHz, and seemingly resembling the currently described machine in construction. Extensive necrosis and regression was obtained in a series of 21 solid human tumours of the lung, colon, kidney and in cancers of the head and neck following treatment at 46–50°C for 30 min. The heating was usually repeated for up to as many as 9 sessions. Tumour temperature was 8–10°C higher than that of normal adjacent tissue in 3 patients in whom this could be measured. The blood flow through 6 human tumours (4 colon, 1 lung, 1 kidney), measured *in vitro*, was only 1–15% of that through the adjacent normal organ. These authors believe the selective RF heating of tumours can be accounted for in terms of decreased blood flow, which would inhibit rapid heat dissipation. The present results with large intramuscular Yoshida tumours and normal leg muscle exposed to the same RF field (Fig. 4, Table 1) do not support this view.

It is known that the resistance offered to the passage of an electric current by a composite electrolyte-dielectric like a living body cannot be explained merely by Ohm's Law [31, 33, 34]. Nevertheless, tissue resistance is a major factor in high frequency heating, which in general follows the ordinary laws of electric heating [31, 33, 34]. Since the tissue resistance, as measured under standardized conditions, was not significantly affected by the presence of the tumour (Table 1), the differential heating of tumour and muscle may reflect a better blood flow through the tumour and a greater ability to contend with the increased heat load.

LeVeen *et al.* [38] also treated Brown–Pearce tumours in rabbits and an unspecified ascites carcinoma in mice by RF heating, but details of the work are totally lacking. It is stated that the temperature was raised to "7–9°C above that of the surrounding tissue" and "these tumours were rapidly and completely necrosed". We have been unable to confirm the results of LeVeen *et al.* in relation to ascites carcinoma in mice. All normal mice heated to an intra-abdominal temperature of 42, 43 or 44°C died rapidly, with haemorrhagic necrosis of the small bowel, and there was no evidence that tumour-bearing animals had increased tolerance to the elevated temperature. The Ehrlich ascites cells were sensitive *in vitro* to the temperatures used, there being a total inhibition of respiration after 4 hr in Warburg flasks at 42°C, or after 2 hr at 43°C; anaerobic glycolysis was not affected at these temperatures (the technique of Warburg manometry and the buffers used were as detailed in [39]). Fifty years ago, Westermark [31] reported extensive coagulation of the abdominal viscera in rats following high-frequency heating of tumours at temperatures over 45°C, and with the electrodes placed on opposite sides of the animal body. The present results with the MC7 sarcoma show that it is feasible to heat superficial tumours to 45°C with electrodes of unequal size "coning", provided the choice of electrode size is such as to selectively heat the tumour and yet prevent the body temperature rising to 42°C by effective dissipation of the current deep to the tumour.

The present method is the best technique of local hyperthermia we have yet tested for tumours in animals. It is easy to perform, and high temperatures with homogeneous tumour heating can be achieved in a reliable and controlled manner, enabling a 100% regression rate to be obtained in heat sensitive tumours. The generated heat seems to act in a manner similar to other forms of heating, e.g. waterbath immersion, since tumour regression after curative heating at a given temperature follows a similar time course [2], and regression of a heated tumour is accompanied by disappearance of tumour elsewhere in the host (the so-called "abscopal" response [40]).

We previously reported rapid death from elevated body temperature in rats bearing 10 ml intramuscular Yoshida tumours heated at 42°C for 1 hr [21]. The animal deaths following heating of large leg tumours at 45°C for 30 min or 50°C for 15 min, and in which the intra-abdominal temperature usually remained below 40°C, remain unexplained. It is presently believed that radiofrequency currents produce their effects solely by heat production within the tissues, and that any damage produced has a purely thermal basis [34, 36].

In relation to the failure to cure the D23 tumour at temperatures up to 45°C, it is of note that this carcinoma is only weakly antigenic [26], while both the Yoshida tumour [41] and the MC7 sarcoma (27) elicit a marked immunological response on the part of the host. As far back as 1927, Westermark [31] believed a host immune response to be involved in tumour cure after local hyperthermia, and Johnson [32] suggested that the Jensen sarcoma was more sensitive to heat than the Walker tumour because it could induce immunity in rats. More recent workers have also adduced circumstantial evidence that tumour regression and host cure following local heating may imply participation of the immune system [1, 2, 8, 22, 23, 42].

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E.O.R.T.C. Protocol for the Therapy of Metastatic Soft Tissue Sarcoma,* A Randomized Trial†

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1. BACKGROUND AND INTRODUCTION

UNTIL recently chemotherapy in metastatic soft tissue sarcoma has given poor results. Since a few years adriamycin (ADM) as a single agent in these tumors has given higher remission rates than obtained with any other drug [1, 2].

Gottlieb's results [3–6] and those of others [7], with combination therapy in soft tissue sarcoma are encouraging. In broad phase II studies during 1970–1971 adriamycin and 5-(3,3-dimethyl-l-triazeno)imidazole-4-carboxamide (NSC-45338) both proved to have definite activity when given as single agents in soft tissue sarcoma [1, 2, 8]. These effects were obtained despite extensive previous therapy with surgery, radiotherapy and the commonly used chemotherapeutic agents vincristine (VCR), actinomycin D (ACD) and cyclophosphamide (CPA). The remission rates for ADM and NSC-45338 (DIC) in these studies were 29 and 15% respectively. His studies also show that ADM is the most active single drug agent and that it can be combined well with other agents. Regression rates of single and combination therapy in soft tissue sarcoma are shown in Table 1. It can be seen that the effects of ADM and DIC are additive [5], while this has been shown in experimental data as well [9]. The two drugs can be given together with little increase in toxicity. Addition of VCR appears to act independently at essentially the same level as DIC. The median duration of remission of combination treatment has been significantly longer (16 months for ADM + DIC).

In a pilot study performed in 1974–1975 within the Soft Tissue Sarcoma Working Party ACD has been introduced instead of VCR. However, toxicity of this 4-drug combination was unacceptable.

Actinomycin D had been introduced in the combination treatment because this drug is effective in single drug treatment of soft tissue sarcoma. Because of the toxicity the schedule has been changed. A single dose treatment with the 4-drug CYVADIC combination described by Gottlieb will be compared with a cycling schedule. With the alternating administration ADM can be given for a longer period of time, while resistance might be built up less rapidly.

2. PURPOSE OF STUDY

The purpose of this trial is to investigate the remission rate and duration of remission and survival of two schedules of 4-drug treatment in soft tissue sarcoma, a single dose schedule and a cycling schedule. The proposed alternating schedule has not been studied before in soft tissue sarcoma, but the four drug combination has been studied previously [4]. The second objective of this study will be to verify these results.

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Table 1. *Remission rate of single and combination therapy of ADM and DIC* with VCR† and CPA‡*

	All sarcomas	Sarcomas—(Ewing + osteo)
ADM (pooled data, Gottlieb)	36/131 (29%)	16/76 (21%)
DIC	10/67 (15%)	10/66 (16%)
ADM + DIC (SWCCG)	85/200 (42%)	71/167 (43%)
ADM + DIC + VCR (SWCCG)	19/40 (48%)	18/35 (51%)
	45/107 (42%)	
ADM + DIC + VCR + CPA (SWCCG)	75/136 (55%)	

*Imidazol carboxamide.
†Vincristine.
‡Cyclophosphamide.
From Gottlieb. Adriamycin Symposium Brussels, 1974.

3. SELECTION OF PATIENTS

3.1 *Conditions of patient eligibility*

1. Eligible patients must have confirmed residual or metastatic soft tissue sarcoma.

The cell types which are included are summarized in Section 10.

2. All patients should have measurable and evolutive disease. Osseous lesions and pleural effusions are not considered as measurable lesions. Progression must be proved within a period of 2 months before the beginning of the treatment, but if it has been proven in a shorter period of time, the patient can enter on study.

3. All patients previously treated by radiation with progressive and measurable growth outside the field of radiation may be accepted for the present study.

4. The patient must be inoperable without other definitive therapy available.

3.2 *Conditions for patient exclusion*

1. Patients 14 years old or younger.
2. Patients above 75 years of age.
3. Patients with overt psychoses or marked senility.
4. Performance status according to Karnofsky under 50 (see Table 2).
5. Active second tumor or serious concomitant disease.
6. Acute intercurrent complications such as infection or post surgical complication.
7. Previous treatment with ADM, DTIC, VCR or CPA.
8. Congestive heart failure.
9. Central nervous lesions including metastases.
10. Unfavourable haematologic status: leukocytes < 4000 per mm^3, thrombocytes $< 100{,}000$ per mm^3.

4. DESIGN OF TRIAL

4.1 Patients will be randomized to receive either the S_1 single dose schedule or the S_2 cycling schedule. Patients will be stratified according to cell type since there are differences in remission rates between the histologic subtypes and also according to age (15–59 years, 60–75 years). No stratification for histologic grading will be done (Table 3).

4.2 Treatment should be administered, if possible, for a minimum of 8 weeks. In responders or in patients with static disease, the dose schedule should be continued for at least 2 years. Patients go off study upon progression and should be followed until death whenever possible.

5. THERAPEUTIC REGIMENS

5.1 For patients between 15 and 59 years of age, the schedule will be

S_1: ADM 50 mg/m^2, Day 1
DTIC 250 mg/m^2/day, Days 1–5
CPA: 500 mg/m^2, Day 1
VCR: 1·5 mg/m^2 (top dose 2 mg), Day 1
This course is repeated every 4 weeks.

S_2: ADM: 50 mg/m^2, Day 1 plus
DTIC 250 mg/m^2/day, Days 1–5;
CPA: 1200 mg/m^2, Day 1 plus
VCR 1·5 mg/m^2 (top dose 2 mg),
Day 1
Cycled dose: every 4 weeks, alternating (ADM + DTIC) and (CPA + VCR).

5.2 For patients between 60 and 75 years of age, the schedule will be

S_1: ADM: 35 mg/m^2, Day 1
DTIC: 170 mg/m^2/day, Days 1–5
CPA: 330 mg/m^2, Day 1
VCR: 1·0 mg/m^2, Day 1
This course is repeated every 4 weeks.

S_2: ADM: 35 mg/m^2, Day 1 plus
DTIC, 170 mg/m^2/day, Days 1–5
CPA: 800 mg/m^2, Day 1 plus
VCR, 1·0 mg/m^2, Day 1.
Cycled dose: every 4 weeks, alternating (ADM + DTIC) and (CPA + VCR).

Table 2. Dose modifications in case of leukopenia and/or thrombocytopenia

	Thrombocytes		
	≧ 100,000	75,000–100,000	≦ 75,000
Leukocytes ≧ 4000	100%	50%	—
2000–4000	50%	50%	—
≦ 2000	—	—	—

Table 3. Drug schedule

		Age	
		15–59	60–75
S_1:	Adriamycin, Day 1	50 mg/m²	35 mg/m²
	DTIC, Days 1–5	250 mg/m²/day	170 mg/m²/day
	Cyclophosphamide, Day 1	500 mg/m²	330 mg/m²
	Vincristine, Day 1	1·5 mg/m² (top dose 2 mg)	1·0 mg/m²
	This course is repeated every 3 weeks.		
S_2:	Adriamycin, Day 1 plus	50 mg/m²	35 mg/m²
	DTIC, Days 1–5	250 mg/m²/day	170 mg/m²/day
	Cyclophosphamide, Day 1 plus	1200 mg/m²	800 mg/m²
	Vincristine, Day 1	1·5 mg/m² (top dose 2 mg)	1·0 mg/m²
	Cycled Dose: every 3 weeks, alternating (ADM+DTIC) and (CPA+VCR).		

Table 4. Dose modifications of ADM in hepatic dysfunction

Bilirubin	BSP-retention	% of normal dose
< 2·0 mg/100 ml	< 12%	100%
2–3 mg/100 ml	12–18%	50%
> 3 mg/100 ml	> 18%	25%

5.3 *Duration of therapy*

ADM will be administered to a total dosage of 550 mg/m². After reaching this dosage, therapy will be continued with DTIC, VCR and CPA at the same doses. No dose escalation is permitted. In patients still showing disease progression after a treatment period of 6 weeks, therapy has to be discontinued. In responders or in patients with static disease the dose schedule will be continued for at least two years. Treatment for patients going off study due to progression will be determined individually by each institution. However, it is recommended that patients failing in S_2 will be crossed over to S_1. It is suggested to treat patients failing on S_1 by doubling the S_1 doses (if facilities for isolation of patients are available).

5.4 *Drug formulation and procurements*

5.4.1 *Supplied.* ADM: vials containing 10 mg; DTIC: vials containing 100 and 200 mg; VCR: vials containing 1 mg; CPA: vials containing 100, 200, 500 or 1000 mg as a powder.

5.4.2 *Storage.* ADM: refrigerator 4°C; DTIC: deep freeze −20°C; VCR: refrigerator 4°C; CPA: refrigerator, keep dry.

5.4.3 *Methods of administration.* All four drugs should be introduced i.v. (pushdose).

5.5 *Supportive therapy*

No radiotherapy should be given to patients in the trial, except for palliative treatment of lesions of the skeleton.

5.6 *Nausea and vomiting*

Phenothiazine should be administered before giving medicaments.

6. TOXICITY AND DOSE MODIFICATIONS

6.1 Dose modifications of adriamycin should take place with hepatic dysfunction according to Table 4. This dose

should also be applied with development of liver dysfunction after start of treatment. Dose modifications of adriamycin should not be applied with renal dysfunction.

6.2 If at 4 weeks the leukocytes are between 2000 and 4000 and/or the thrombocytes between 75,000 and 100,000 the dose will be adjusted according to Table 2. If at 4 weeks leukopenia and/or thrombocytopenia persist below these levels, treatment will be postponed a maximum of 3 weeks. If a cycle has to be postponed more than 3 weeks, the patient should be dropped from the trial. At the moment (within 4–6 weeks from the beginning of the last cycle) that the leukocytes and thrombocytes have recovered to levels above 2000 and 75,000 respectively, treatment can be recontinued at 50% of the initial dose. In this case the dosage of subsequent cycles will be maintained at 50% of the initial dose even if leukocytes and thrombocytes recover to normal levels within 3 weeks.

7. PRETREATMENT STUDIES

7.1 *History*

a. Age
b. Sex
c. Date and nature of first symptoms
d. Date of initial diagnosis and how made
e. Prior treatment (dates, methods, nature and duration of response).

7.2 *Physical examination*

a. Ambulatory status using Karnofsky's criteria
b. Height, weight and surface area
c. Description and measurements of soft part metastases with a suitable caliper
d. Photographs of the soft tissue lesions. (optional),

7.3 *Laboratory studies*

a. Complete blood count
b. Serum creatinine, Na, K, Ca
c. Serum uric acid
d. Bilirubin, alkaline phosphatase, transaminases, serum proteins and electrophoresis
e. Urinalysis.

7.4 *X-thorax*

7.5 *Bone*

Skeletal X-ray survey including following bones: skull lateral and AP views, pelvis AP view, lumbar, dorsal, cervical spine lateral and AP views.

7.6 *Isotopic scintigraphy*

Liver scan; skeletal scan: skull, spine and pelvis.

7.7 *Electrocardiogram*

8. FOLLOW-UP STUDIES

8.1 Every week during the first 8 weeks and then every 5 weeks thereafter: 7.3a.

8.2 Every 4 weeks: 7.2a, 7.2b, 7.2c, 7.2d.

8.3 Every 8 weeks: 7.3b, 7.3c, 7.3d, 7.3e, 7.4.

8.4 Every 6 months: 7.5, 7.6, unless indicated earlier either on a medical indication or for determination of a complete remission.

8.5 In case of objective tumor response: pre-treatment studies except 7.1.

8.6 In case of toxicity: follow-up of the toxicity until complete recovery.

8.7 Every side-effect of therapy has to be recorded: anorexia, nausea, vomiting, mouth ulcerations, alopecia, haemorrhagic cystitis, hepatic dysfunction.

8.8 Complementary studies may be performed provided they do not interfere with or bias the results of the studies required by the protocol. Immunological studies are optional.

9. DEFINITION OF RESPONSE

Only objective tumor response criteria are used in these studies. These are the following:

9.1 A complete remission is defined as the disappearance of all symptoms and signs of soft tissue sarcoma for a minimum of 4 weeks.

9.2 A partial remission is defined as a significant decrease in size in at least 50% of all lesions for a minimum of 4 weeks while the remainder are static. In the case of accurately measurable lesions, such a decrease is defined as a reduction by 50% of the product of the two largest tumor diameters. For lesions that do not lend themselves to accurate measurement, the reduction should be at least three-fourths of the estimated volume. Changes in body chemistry and improvement in hemodoiesis cannot be construed as criteria of objective remission.

9.3 No change is recorded for a patient when no new lesions appear and no lesions increase in size; decreases in lesion size, if any, are not sufficient to indicate a partial remission.

9.4 Progression occurs when any lesion increases in size or any new lesions appear, regardless of what the response of the other lesions may have been.

10. REGISTRATION AND RANDOMIZATION OF PATIENTS

A patient is registered and randomized after the local pathology review by telephoning to the E.O.R.T.C. Data Center (tel: Brussels 538.65. 33) from 9.00 a.m. to 5.00 p.m., Monday through Friday. The date of registration is the date of making this telephone call. At this time the following information is requested:

1. Protocol number (62761).
2. Patient's age (15–59 or 60–75 yr).
3. Cell type (see below).
4. Patient's name.
5. Institution's name.
6. Physician's name.
7. Caller's name.

The treatment assigned by randomization will then be given.

If registration by telephone is not possible, patients may also be registered by telex: 22773, or by mail:

E.O.R.T.C. Data Center,
Institut Jules Bordet,
1, rue Héger-Bordet,
1000 Bruxelles, Belgium,

by including the information requested above. Stratification will take place on the patient's age (15–59 or 60–75 yr) and on the following cell types:

1. Angiosarcoma (haemangioendotheliosarcoma + haemangiopericytoma)
2. Fibrosarcoma
3. Leiomyosarcoma
4. Liposarcoma
5. Neurofibrosarcoma
6. Rhabdomyosarcoma
7. Synovial cell sarcoma
8. Undifferentiated sarcoma
9. Mesothelioma
10. Unclassified or miscellaneous.

Thus, Kaposi's sarcoma, chondrosarcoma, and osteosarcoma, will be excluded. No stratification will be done on extent of disease or anatomic staging.

11. CENTRAL PATHOLOGY REVIEW

There will be a central pathology review with grading of the histology by the Department of Pathology, University Hospital, Utrecht, The Netherlands. The following material and information is requested:

11.1 Clinical data: age, sex, localization of tumor, number of samples taken for histopathological exam, original macro and micro report.

11.2 Six unstained sections of each sample (or paraffin blocks, by preference tumor tissue in fixation fluid).

11.3 The central Pathology Review would be very grateful to receive tumor tissue, fixed or embedded, for electronmicroscopy. It can be processed and studied by the University Hospital in Utrecht.

11.4 If additional biopsies during the course of the study are done or if an autopsy is performed, the Central Pathology Review would be very interested to study this material as well.

12. SUBMISSION OF FORMS

All forms are to be sent in duplicate to the E.O.R.T.C. Data Center, Institut Jules Bordet, 1, rue Héger-Bordet, 1000 Bruxelles, Belgium.

The schedule for submission of forms is as follows:

1. *On-study form (Form II)*

Send within one week of the patient's entry on study (randomization).

2. *Chemotherapy form (Form V)*

Send after completion of each 4 week cycle of chemotherapy.

3. *Flow sheet (Form VII)*

The first flow sheet should be filled out prior to the first cycle of chemotherapy and sent with the on-study form. It should also be filled out at 8 weeks, 4 months, and then every 3 months thereafter.

4. *Measurement form (Form VIII)*

The schedule is the same as for the flow sheet. The first measurement form should be filled out prior to the first cycle of chemotherapy

and sent with the on-study form. It should also be filled out at 8 weeks, 4 months, and then every 3 months thereafter. The location of the lesion is identified by the grid on page 2 of the measurement form. The location of any lesion is identified by a letter (horizontal axis) followed by a 2 digit number (vertical axis). Initially the coordinates relating to the center of the lesion should be used. When following a lesion, the same coordinates must always be used to identify the lesion even if the lesion changes in size and shape. In the case of multiple lesions in one area, the largest lesion should be indicated on the form.

5. *Summary form (Form IX)*

To be sent in whenever the patient goes off study for any reason.

Patients who go off study while still alive should be followed until death whenever possible and the Data Center notified of the date of death, cause of death, and localization of disease if the cause of death is malignant disease, upon the death of the patient.

13. STATISTICAL CONSIDERATIONS

Three hundred patients will be entered on study and randomized to receive either the single S_1 or cyclic S_2 dose schedule. While a remission rate of approximately 50% is expected on each arm, 150 patients on each arm is sufficient to detect a difference of 15% in the two response rates. ($\alpha = 0{\cdot}05$, $\beta = 0{\cdot}20$). If the remission rate is approximately 50% then 75 responding patients on each arm is sufficient to detect a ratio of 1·5:1 in the mean (or median) length of the time to progression of the two treatments if all responding patients are followed until progression ($\alpha = 0{\cdot}05, \beta = 0{\cdot}20$) This assumes that the time to progression follows an exponential distribution.

The expected duration of the trial depends on the number of (responding) patients entered each year and the distribution of the time to recurrence. If 100 evaluable patients are entered each year and 50% respond, then it will take 3 years to enter the required number of patients. However, to do an analysis at the error rates given above, the length of the trial will depend on the length of the longest time to progression. If the median length of remission is one year on S_1, then in order to detect a median length of remission of 1·5 years on S_2, the shortest duration of the trial will occur if patients are entered for 4·7 years. After this time a definitive analysis can be made.

14. ADMINISTRATIVE RESPONSIBILITIES

This study is a joint effort between the E.O.R.T.C. Soft Tissue Sarcoma Cooperative Group:

Nederlands Kanker Instituut, Amsterdam: Dr. R. Somers.
Rotterdamsch Radiotherapeutisch Instituut, Rotterdam: Dr. R. Treurniet, Dr. J. H. Mulder.
Academisch Ziekenhuis, Leiden: Dr. A. T. van Oosterom.
Academisch Ziekenhuis, Utrecht: Dr. H. M. Pinedo.
Radboud Ziekenhuis, Nijmegen: Dr. Th. Wagener.
Ziekenhuis Westeinde, 's-Gravenhage: Dr. G. Booy.
Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse, Villejuif, France 94800: Prof. C. Jasmin.
Istituto Nazionale per lo Studio e la Cura Dei Tumori, Milano, Italy: Dr. G. Bonadonna.
Innere Klinik und Poliklinik (Tumorforschung) der Ruhruniversität Essen, Germany: Prof. Dr. C. G. Schmidt.
Zentrum für Innere Medizin, Robert Bosch-Krankenhaus, Stuttgart, Germany: Prof. Dr. W. Wilmanns.
Christie Hospital, Cancer Research Campaign, Manchester, United Kingdom: Prof. D. Crowther.
Institut Jules Bordet, 1000 Bruxelles, Belgium: Dr. Y. Kenis and Dr. M. Staquet.
Swiss Group for Clinical Cancer Research, 62 rue de Carouge, 1205 Genève: Dr. H. J. Senn.
University Hospital, 9000 Ghent, Belgium: Dr. A. De Schryver.

Study Coordinator

Dr. H. M. Pinedo, Oncology Unit,
Department of Internal Medicine,
Academisch Ziekenhuis, Utrecht,
The Netherlands.
Telephone: 030–379111 or 372202.

Central Pathology Review

Professor G. Bras and Professor J. V. Unnik,
Department of Pathology,
University Hospital,
Utrecht, The Netherlands.
Telephone: 030–379111.

Statistical Service

E.O.R.T.C. Data Center,
Institut Jules Bordet,
Rue Héger-Bordet 1
1000 Bruxelles, Belgium.
Telex: 22772
Telephone (02) 538.65.33.

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Letter to the Editor

Mammalian mRNA Translation of the Liver Ribosome Subunits Isolated from Dimethylnitrosamine-Treated Mice

A. DELPINO and U. FERRINI

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THE CARCINOGENIC compound dimethylnitrosamine (DMN) displays strong hepatotoxic effects in several mammalian species when given in a single dose ranging from 30 to 100 mg/kg of body weight.

The main effect, within 2 hr after treatment, is a severe reduction of the liver protein synthesis, parallel to a progressive polysomal disaggregation and increase of 80 S monomer fraction.

A number of experimental evidences seems to indicate that this inhibition is caused by an impairment of the ribosome recycling and, therefore, of the initiation step of the protein synthesis. The mechanism of this effect, however, is still unknown.

DMN-generated monomers have been found to have many of the features typical of monomers of run-off type, rather than of monomers produced by the mRNA breakdown [1, 2]. Previous observations have also shown that these monomers and their constitutive subunits are fully competent for the poly-U directed polyphenylalanine synthesis [2, 3]. It is well known, however, that such an assay cannot be adopted as a test for controlling the ribosome capacity to synthetize proteins by means of the specific initiation mechanism, i.e., by the recognition of the initiation codon on natural eukaryotic messenger.

In order to clarify this point, we have tested purified subunits, isolated from the DMN-

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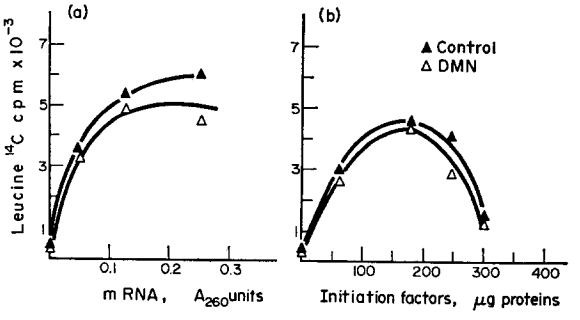

Fig. 1. Rabbit globin mRNA-directed protein synthesis with ribosomal subunits isolated from control (▲) and DMN-treated (△) animals.

Treated mice received an i.p. injection of 50 mg/kg b.w. of redistilled DMN 3 hr before killing.

Liver ribosome subunits were isolated by high salt (300 mM KCl and 3 mM $MgCl_2$) gradient centrifugation directly from detergent treated postmitochondrial supernatant.

Crude initiation factors and globin mRNA were prepared from rabbit reticulocyte ribosomes according to published methods (4, 8).

Incorporation was carried out in 0, 1 ml reaction mixture containing 0·16 A_{260} units of 40 S + 0·40 A_{260} units of 60 S ribosomal subunits; 30 μl of pH 5 enzyme (about 600 μg of protein); 0·1 μmole of ATP; 0·05 μmole of GTP; 2 μmole of creatine phosphate; 0·25 units of creatine kinase; 2 nmole of each L-amino acid (minus leucine); 0·25 μCi of ^{14}C-leucine (324 mCi/mM); 1·3 μmole of Tris–HCl (pH 7·6); 7 μmole of KCl; 0·45 μmole of $MgCl_2$; 0·65 μmole of β-mercaptoethanol. In panel A the amount of protein initiation factors was 150 μg and the amount of rabbit globin mRNA was made variable. In panel B the amount of mRNA was 0·15 A_{260} units and the amount of protein initiation factors was made variable. Incubation was carried out for 30′ at 37°C.

Reaction was stopped by the addition of 3 ml of 10% cold CCl_3COOH. Samples were heated 15′ at 90°C, then collected onto glass fibre filters and washed three times with cold 5% CCl_3COOH.

generated monomers, in an *in vitro* system programmed with rabbit globin mRNA and supplemented with heterologous initiation factors.

As shown in Fig. 1A and 1B, DMN-subunits are able to translate on natural mammalian mRNA with the same efficiency of the corresponding control subunits.

In the presence of a fixed amount of initiation factors (Panel A) both control and DMN-subunits are saturated by the same quantity of mRNA (0·15 A_{260} units of globin mRNA for 0·16 A_{260} units of 40 S and 0·40 A_{260} units of 60 S).

An identical behaviour between control and DMN-subunits is also evident when the synthetic capacity is tested by varying the concentration of initiation factors at fixed amount of globin mRNA (Panel B). The maximum activity for 0·5 A_{260} units of reconstituted ribosome couples is attained when 200 μg protein of unfractionated ribosome KCl wash are present; by increasing the amount of the KCl extract the protein synthesis progressively decreases, owing to the presence of inhibitory factors in such crude extract [4]. In the absence of ribosome KCl wash, the incorporation activity is strongly reduced, as expected for ribosome subunits which, after isolation in high salt gradients, are known to be completely devoid of their endogenous initiation factors.

On the basis of these results we can assume that, within the first 3 hr after DMN injection, the protein synthesis capacity of the subunits derived from DMN-generated monomers is unchanged and therefore the ribosomes *per se* do not play any significant role in the inhibition of the protein synthesis following a toxic dose of DMN.

Our results are in agreement with the data of Nygard and Hultin [5] which demonstrated that monomers prepared from DMN-treated rats are able to translate on heterologous mammalian mRNA, when supplemented with homologous unfractionated initiation factors.

Anyhow, the observation that these ribosomes retain a fully capacity to synthetize proteins badly correlates with the fact that, up to the first hours, their 18 and 28 S rRNAs are methylated at a very fast rate and with the highest activity among the various cellular components [6]. In this connection it is worthy to mention the fact that the mammalian ribosomes can support within certain limits a nucleolytic attack, without loosing their capacity to synthetize proteins [7]. It is possible, therefore, that the alkylation of the ribosome may produce a functional defect whenever the number and the distribution of alkyl groups on the nucleophylic centers attain a critical level.

According to this hypothesis, we have observed a constant slight reduction on the activity of the DMN-derived subunits [2]; this fact can be related to the presence of a number of a fully inactivated particles.

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Letter to the Editor

Nuclear DNA Polymerase-α and Replicative Potential in Mammalian Cells*

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DNA POLYMERASE-α has been investigated as a potential marker to distinguish proliferative and nonproliferative cells [1]. This enzyme copies activated DNA at a high rate and comprises 80–90% of the total cellular polymerase. It has a sedimentation coefficient of 6–8S and a molecular weight of 110,000–220,000 daltons [2], although it has also been suggested that the enzyme may be composed of two or more subunits of 55,000–87,000 daltons [3]. The α-polymerase activity, unlike the β-polymerase, can be inhibited with agents that bind sulfhydryl groups, such as *p*-chloromecuribenzoate (*p*-CMB) and *n*-ethylmaleamide [4, 5]. Although the α-polymerase can be isolated from the cytosol of cells [1, 6, 7], its activity has also been demonstrated in the nucleus [7, 8]. Unlike β-polymerase, α-polymerase levels in the nucleus have been shown to correlate well with the proliferative status of the cell population [8–11].

Although most DNA polymerase assays utilize liquid scintillation counting techniques to detect ^{3}H-thymidine triphosphate (^{3}H-TTP) incorporation into activated primer-template, an autoradiographic, slide assay has been described in which DNA polymerase activity in isolated cell nuclei is measured utilizing the cells own DNA as primer-template [12]. Utilization of this technique (primer available, DNA polymerase or PDP assay) permits enumeration of the fraction of cells in which both DNA polymerase and endogenous available primer-template are present in the same nucleus. Other more recent studies have shown that the fraction of PDP labeled nuclei, for a variety of experimental tumors, gives a good estimation of the tumor growth fraction as measured by more classical methods [13]. In the present studies the PDP index and mean grain count were determined in the presence of the sulfhydryl binding agent (*p*-CMB) to determine if the enzyme component of the PDP assay is the DNA α-polymerase.

In these studies, touch imprint preparations of solid tumors and dry film smears from spleen cell suspensions were subjected to PDP assays as previously described [12, 13]. Briefly, the slide preparations were dipped in 0.25% agar (to remove cytoplasm, leaving nuclei adherent to the slide), air dried and fitted with a glass incubation chamber. The slides were incubated for 45 min at 37°C with a mixture containing the four nucleotide triphosphates, including 5μCi per chamber of ^{3}HTTP at specific activity 54Ci/mM.

The incubation mixture, optimized with respect to Mg^{2+}, was buffered with Tris-HCL (pH 7.4) and included ficoll as a stabilizing agent. In addition, duplicate slides for each tumor were treated in a similar manner except for addition of various concentrations of *p*-CMB to the reaction mixture at the beginning of the incubation period.

Following incubation the slides were rinsed thoroughly in tap water, then triple distilled water and air dried. The slides were subjected to autoradiography with Kodak NTB-2 liquid photographic emulsion as previously described

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[14]. All exposure times were 7 days. Labeling indices were determined on at least 500 cells per tumor, and mean grain counts determined on at least 100 cells per sample. Background levels determined in adjacent acellular areas of each sample were consistently less than 2 gr per equivalent cell area and subtracted from the mean grain count. For ease of comparison, the mean grain counts in *p*-CMB samples were expressed as a percent of the untreated controls.

The PDP index, the ^{3}H-TdR labeling index, and the cell production rates were determined for a variety of experimental tumors, including spontaneous C3H/He mammary tumors, "fast" and "slow" line mammary tumors, the 13762 transplantable rat mammary tumor, a transplantable rat glioma, the T1699 transplantable mouse mammary tumor and the 2 day S-180 ascites tumor. The ^{3}H-thymidine labeling indices and DNA synthesis times were determined as described previously [14], and the cell production rate calculated by the method of Steel, $K_p = 0.693/(\lambda T_S/{}^3\text{HTdR LI})$ [15].

Figure 1 shows the results from studies with the 13762 tumor in which the PDP index was 69.1%, the T1699 tumor with a 47.6% PDP index and normal spleen cells from SJL/J mice with an average PDP index of 16.3%. The addition of various concentrations of *p*-CMB results in a quantitative inhibition of ^{3}HTTP incorporation. Regression lines fit by a linear least square method show that the 13762 tumor

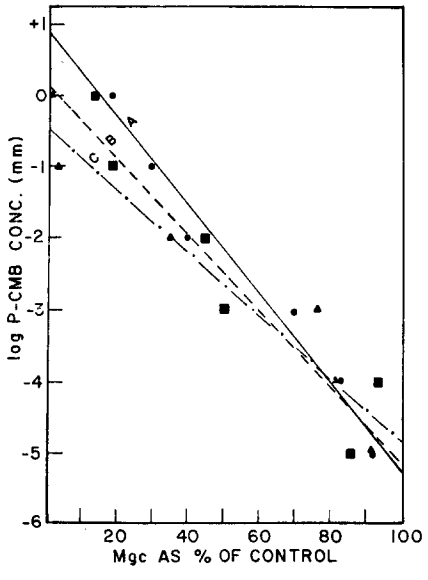

Fig. 1. The effect of increasing p-*CMB concentration on* ^{3}*H-TTP incorporation into isolated cell nuclei, as measured by the mean grain count (mgc) in autoradiograms. For ease of comparison the mgc's were normalized to % of controls untreated with* p-*CMB.* —●—, *13762 rat mammary tumor (A);* – – –■– – –, *T1699 mouse mammary tumor (B);* —·▲·—, *normal spleen cells (C).*

required the greatest concentration of *p*-CMB for complete inhibition, while spleen cells required the least. The concentration for 50% inhibition was calculated at 7.00×10^{-3}mM, 3.17×10^{-3}mM and 2.69×10^{-3}mM *p*-CMB for the 13762, T1699 and normal spleen cells respectively. The results suggest that the PDP assay measures the presence of DNA α-polymerase and that, on an individual basis, the average amount of α-polymerase per labeled cell is greatest in the 13762 tumor and lowest in normal spleen cells.

Table 1 shows the PDP and ^{3}HTdR labeling indices and the DNA synthesis times for the various tumors studied. Figure 2 shows the relationship between the PDP index and the ^{3}HTdR labeling index and the PDP index and the cell production rate (K_p) for the tumors

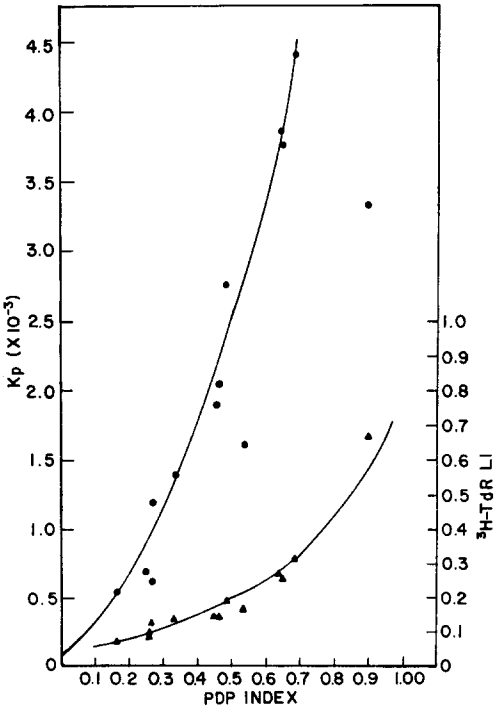

Fig. 2. The relationship between PDP index and cellular production rate (●) and the PDP index and ^{3}*HTdR labeling index (▲) for a variety of experimental tumours (see Table 1 for details).*

listed in Table 1. A line with the equation $y = x/-0.225x+0.333$ was fit by computer ($r = 0.932$) to the data for PDP and K_p, while a line with the equation $y = 4.317 \times 10^{-2}$ $e^{2.833x}$ was similarly fitted to the PDP and ^{3}HTdR LI data ($r = 0{\cdot}968$). The results show a high degree of correlation between the PDP index and both of the cell kinetic parameters reflective of cell proliferation.

In studies in which endogenous DNA was used as primer-template, nuclear DNA poly-

Table 1. Cell kinetic parameters and PDP indices for a variety of experimental tumors

Tumor	Host	Age (Days)	Site*	PDP Index	^{3}HTdR LI	T_s (hr)
C3H/HeJ Spontaneous mammary	C3H/He Retired breeders	Variable	s.c.	0.166 ± 0.004 (91)†	0.069 ± 0.05 (23)	10.9 ± 0.2 (10)
A-67§ "slow line"	C3H/He	15	s.c.	0.268 ± 0.009 (10)	0.078 ± 0.007 (10)	11.5 ± 0.2 (10)
Glioma‖	Fisher 344 rat	14	s.c.	0.272 ± 0.010 (4)	0.125 ± 0.001 (4)	9.6 ± 0.2 (4)
		14	i.c.*	0.337 ± 0.034 (4)	0.143 ± 0.007 (4)	9.4 ± 0.3 (4)
13762 Mammary¶	Fisher 344 rat	35	s.c.	0.464 ± 0.008 (5)	0.144 ± 0.016 (5)	5.9 ± 0.2 (5)
T1699 Mammary	DBA Mouse	24	s.c.	0.471 ± 0.051 (5)	0.142 ± 0.052 (5)	5.3 ± 0.2 (5)
T1699 Mammary	DBA Mouse	23	s.c.	0.489 ± 0.026 (5)	0.190 ± 0.025 (5)	5.3 ± 0.3 (5)
S-102§ "fast line"	C3H/He	15	s.c.	0.540 ± 0.016 (10)	0.172 ± 0.009 (10)	9.5 ± 0.2 (10)
13762¶	Fisher 344 rat	25	s.c.	0.645 ± 0.035 (5)	0.276 ± 0.018 (5)	5.6 ± 0.2 (5)
T1699	DBA Mouse	14	s.c.	0.653 ± 0.015 (5)	0.262 ± 0.011 (5)	5.4 ± 0.2 (5)
13762¶	Fisher 344 rat	14	s.c.	0.691 ± 0.019 (5)	0.310 ± 0.021 (5)	5.7 ± 0.1 (5)
S-180	C3H/He	2	Ascites	0.899 ± 0.020 (5)	0.677 ± 0.059 (5)	13.7‡

*s.c., subcutaneous; i.c., intracranial.
†Mean ± 1 S.E.M. (N).
‡From PLM data of Schiffer *et al.* [13].
§Obtained from Dr. L. Dethlefson.
‖ Obtained from Dr. R. Selker.
¶Originally obtained from Dr. A. Bogden.

merase activity was shown to be increased during DNA synthesis [9]. In regenerating rat liver, DNA polymerase-α activity in the nucleus increased prior to increases in ^{3}H-thymidine incorporation [8, 10]. Although cytoplasmic polymerase-α activity may be an artifact of the isolation procedure [8], nonproliferating AKR lymphoma cells have low or nonexistent cytoplasmic α-polymerase activity. In unperturbed tumor systems the PDP index has been shown to be a good estimate of the fraction of proliferating tumor cells. Further, in studies with solid tumors [16] and human leukemia [17] the PDP index has been shown to be a responding modality to agents which inhibit DNA synthesis.

A number of studies have also indicated that proliferating and nonproliferating cells may have different levels of primer-template activity [18, 19] and suggest that in assessing proliferative status both endogenous primer-template activity as well as α-polymerase activity are crucial. This may be especially important in perturbed systems where α-polymerase levels alone determined on exogenous primer-template may be misleading in terms of cell proliferation [11].

The results from the present study and others in this laboratory [11, 12] have shown that the PDP assay measures the simultaneous presence of nuclear DNA polymerase and endogenous available primer-template. We now show that the nuclear DNA polymerase is sensitive to *p*-CMB and this suggests that the enzyme is probably DNA polymerase-α. The PDP index, i.e. the fraction of cells with both α-polymerase and endogenous primer-template, correlates strongly with, but is not equivalent to, the ^{3}HTdR labeling index and the cell production rate. Since the PDP index has been demonstrated to be a good estimate of tumor growth fraction [13] we suggest that it could be an important parameter for therapy decisions.

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Europ. J. Cancer Vol. 13, p. 781. Pergamon Press 1977. Printed in Great Britain

Announcements

The Czechoslovak Society for Oncology, commissioned by the Czechoslovak Medical Society J. E. Purkyne is organizing the:

3rd CZECHOSLOVAK CONGRESS OF ONCOLOGY
WITH INTERNATIONAL PARTICIPATION

To be held in Bratislava from 26–29 October 1977.

The European Organization for Research on the Treatment of Cancer is in the process of compiling a directory of European statisticians working in the field of clinical trials in cancer. Statisticians working in this area in Europe are asked to please write to:

Richard Sylvester,
E.O.R.T.C. Data Center,
Institut Jules Bordet,
1 rue Heger-Bordet,
1000 Brussels, Belgium,

requesting information concerning this directory.

Europ. J. Cancer Vol. 13, pp. 782. Pergamon Press 1977. Printed in Great Britain

Recent Journal Contents (1977)

British Journal of Cancer *July*, 1977

Book Reviews

Europ. J. Cancer Vol. 13, pp. 783–784. Pergamon Press 1977. Printed in Great Britain

Papers to be Published

S. HARGUINDEY, W. A. SPEIR, R. C. KOLBECK and E. D. BRANSOME

Alkalotic disequilibrium in patients with solid tumors: rediscovery of an old finding.

S. MADAJEWICZ, J. HARUPPA and J. KAMINSKA

Insulin-induced growth hormone response in patients with uterus carcinoma. I. Endometrial carcinoma.

R. D. RUBENS, P. ARMITAGE, P. J. WINTER, D. TONG and J. L. HAYWARD

Prognosis in inoperable stage III carcinoma of the breast.

REIKO YANAI and HIROSHI NAGASAWA

Effect of progesterone and estrogen on DNA synthesis of pregnancy-dependent mammary tumors in GR/A mice.

L. MORASCA, M. RECCHIA, E. GRAMELLINI, G. BOLIS and C. MANGIONI

Melphalan vs polymelphalanum in ovarian cancer patients resistant to cyclophosphamide: a tentative statistical approach to balance risks and benefits.

H. NEWMAN, LADA VODINELICH and C. W. POTTER

The effects of 1-β-D ribofuranosyl-1,2, 4-triazole-3-carboxamide (Ribavirin) on the transplanted tumours of animals.

K. ALEXANDROV

Effects of inducers and inhibitors of the benzo(a)pyrene hydroxylase of isolated rat liver nuclei and nuclear envelopes on the binding of benzo(a)pyrene to DNA.

BRIDGET T. HILL, L. A. PRICE, S. I. HARRISON and J. H. GOLDIE

The difference between "selective folinic acid protection" and "folinic acid rescue" in L5178Y cells culture.

N. THATCHER, R. J. BARNARD, N. GASIUNAS and D. CROWTHER

Changes in cellular immunity following nephrectomy for localized and metastatic hypernephroma.

M. K. JONES, I. D. RAMSAY, W. P. COLLINS and GAIL I. DYER

Plasma testosterone concentrations in patients with tumours of the breast.

R. J. B. KING, J. L. HAYWARD, S. KUMAOKA and H. YAMAMOTO

Comparison of soluble oestrogen and progestin receptor content of primary breast tumours from Japan and Britain.

LEA CERCEK and B. CERCEK

Application of the phenomenon of changes in the structuredness of cytoplasmic matrix (SCM) in the diagnosis of malignant disorders: a review.

P. V. MAYNARD, A. W. PIKE, A. WESTON and K. GRIFFITHS

Analysis of dehydroepiandrosterone and androstenediol in human breast tissue using high resolution gas chromatography–mass spectrometry.

MADHAV WAGHE and SHANT KUMAR

Demonstration of a Wilms' tumour associated antigen using xenogenic antiserum.

OLLE HEBY, LAURENCE J. MARTON, CHARLES B. WILSON and JOE W. GRAY

Effect of methylglyoxal-Bis (guanylhydrazone), an inhibitor of spermidine and spermine synthesis, on cell cycle traverse.

P. A. MAURICE and C. LEDERREY

Increased sensitivity of chronic lymphocytic leukemia lymphocytes to alkylating agents due to a deficient DNA repair mechanism.

R. HULHOVEN, J. P. DESAGER, G. SOKAL and C. HARVENGT

Plasma levels and biotransformation of infused daunorubicin and daunorubicin–DNA complex in rabbits: a preliminary report.

J. SHEWELL and R. W. DAVIES

Combined therapy of the spontaneous mouse mammary tumour: methotrexate and hyperbaric oxygen irradiation.

Europ. J. Cancer Vol. 13, pp. 785–787. Pergamon Press 1977. Printed in Great Britain

A Message from the Editor

The *European Journal of Cancer* was created in 1965, and the first issue appeared in June of the same year. The founder members are all members of the European Organization for Research on Treatment of Cancer. This is a European and not a national organization. Its objectives are to stimulate and coordinate therapeutic research on cancer in Western Europe. It is a non-governmental multinational body which is supported by private donations and can function effectively because of the support of the individual laboratories and hospitals which are members of the group, with the assistance of a grant from the National Cancer Institute, Bethesda, Maryland, U.S.A., and, more recently, with the contribution from the E.O.R.T.C. Foundation under the chairmanship of Mr. Ronald Grierson, London.

The *European Journal of Cancer* pursues the same objectives as the E.O.R.T.C. It has no restrictive policy as far as the geographical origin of the articles is concerned. However, it has been publishing a majority of manuscripts originating from Western Europe (Fig. 1, Table 1). Of a total number of 1131 published papers, 769 or 68% originate from the countries of the E.E.C. This fact is a justification for the name of the journal, but it is also a realization of the objectives the founder members assigned to the journal: to provide to scientists and clinicians doing cancer research, in this area of the world, a journal with high standards of publication.

Among the E.E.C. countries, the United Kingdom comes first, contributing two and one half the number of articles emanating from any other E.E.C. country.

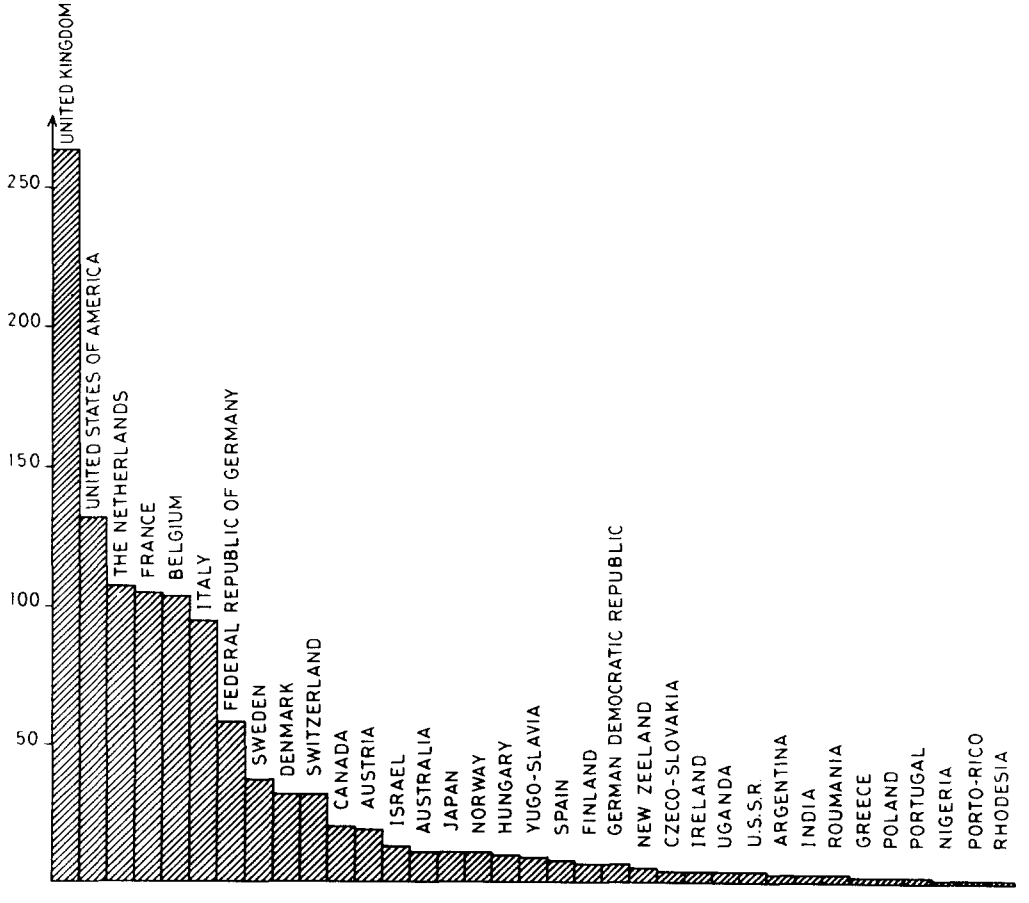

Fig. 1

Table 1. *European Journal of Cancer manuscripts published from* 1965 *through* 1976

United Kingdom	264
United States of America	132
The Netherlands	107
France	105
Belgium	104
Italy	95
Federal Republic of Germany	58
Sweden	37
Denmark	32
Switzerland	32
Canada	20
Austria	19
Israel	13
Australia	11
Japan	11
Norway	11
Hungary	10
Yugoslavia	9
Spain	8
Finland	7
German Democratic Republic	7
New Zealand	5
Czechoslovakia	4
Ireland	4
Uganda	4
U.S.S.R.	4
Argentina	3
India	3
Roumania	3
Greece	2
Poland	2
Portugal	2
Nigeria	1
Porto-Rico	1
Rhodesia	1
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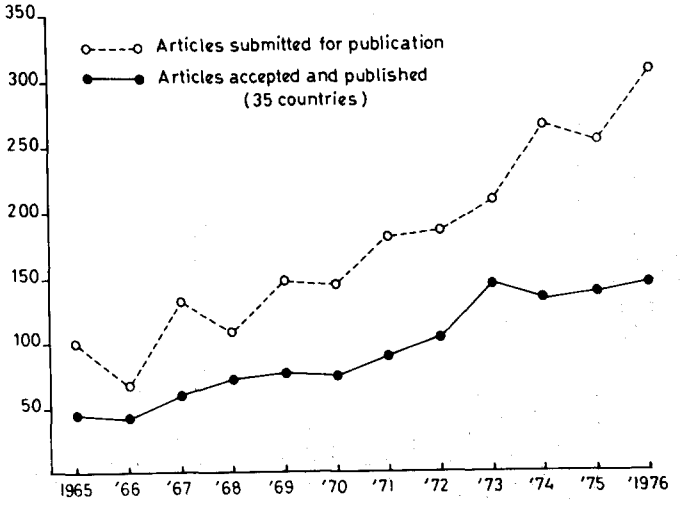

Fig. 2

Table 2. European Journal of Cancer

Year	Manuscripts submitted for publication	Manuscripts accepted and published	Manuscripts published (number of pages)
Quarterly publication			
1965	100	48	330
1966	67	45	388
1967	132	60	438
Bi-monthly publication			
1968	109	71	646
1969	149	77	642
1970	142	75	560
1971	180	88	584
1972	183	105	696
Monthly publication			
1973	209	145	888
1974	274	135	838
1975	255	138	1006
1976	308	144	1028
TOTAL	2108	1131	8044

The Netherlands, France, Belgium and Italy, are at about the same level, despite the difference in size and population among them.

The United States of America represent an important contributor, coming second after the United Kingdom and preceding the other countries. This is a very welcomed contribution and goes a little way towards balancing the numerous publications in United States scientific journals of manuscripts from European Institutes.

Other countries have a smaller participation in the total number of publications, and it is not clear whether this is due to a lesser production or to a preference for national or American journals. The American scientific periodicals remain a formidable attractive force for manuscripts of good quality.

All in all, 35 countries are represented in our Tables of Content. The number of submitted articles has risen from 100 in 1965 to 308 in 1976. The curve (Fig. 2) is a continuous one without major breaks. The curve representing the accepted articles is parallel to the other, extending from 48 published papers in 1965 to 144 in 1976. In number of published pages, the progression is from 330 in 1965 to 1028 in 1976 (Table 2).

The *European Journal of Cancer* has published several symposia and proceedings of meetings. These have usually been published as supplements, and their content should be added to the total published material.

A scientific or medical journal is just as good as its contributors are. If the *European Journal of Cancer* has a good standing in the scientific community, it is due to the quality of the writers who chose to send their manuscripts to our editorial office. But it is also due, in no small measure, to the selfless assistance of the many referees who have accepted to review submitted articles, suggest corrections and improvements and assisted the Editorial Board in making a decision on the acceptability of manuscripts. The list of referees is found in this issue. The Editorial Board is to be thanked also. Finally, the Technical Editor, Miss Leroy, and the Pergamon Press have been the important instruments of our continued expansion.

In the future, the *European Journal of Cancer* will maintain and confirm its policy of publishing papers, the results of research of good quality representing the best that modern medical science can offer.

H. J. TAGNON, M.D.

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R. Van Nie
H. A. Van Peperzeel
L. M. van Putten
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Alkalotic Disequilibrium in Patients with Solid Tumors: Rediscovery of an Old Finding

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Abstract—*Acid-base equilibrium in two groups of cancer patients, one with newly diagnosed bronchogenic cancer and one with various solid tumors, was compared to that of a group of normal volunteers and a group of hospitalized patients with diverse diseases. A consistent tendency toward alkalosis was found in both groups of cancer patients. Similar findings reported in the literature of 40–70 years ago are discussed and the possible biochemical implications of these findings considered. It is our hope that this report of findings obtained with modern technology will encourage studies of the effects of alkalinity in the pathogenesis and progression of cancer.*

INTRODUCTION

THERE are a number of reports in the medical literature of the first half of the twentieth century concerning an apparent association of malignant tumors with systemic alkalosis [1–5]. Furthermore, a few controversial reports suggest the possibility of inhibition of cancer growth by induced systemic acidosis [5–7, 8]. In 1906, Moore and Wilson [4] noted an increase in blood alkalinity in a large series of cancer patients as measured by titration techniques. They suggested that alkalinity was a cause rather than an effect of cancer because of its presence early in the course of the neoplastic process and its persistence after surgical removal of malignant tumors. Similar findings were reported in 1929 by Reding and Slosse [5, 9] using colorimetric methods and once again the suggestion was made that alkalosis might play an etiologic role in cancer. Interpretation of such findings is difficult nowadays not only because of the relatively crude laboratory methodology employed, but also because there is little information on the clinical condition of the patients studied, and no assurance that other causes of alkalosis had been considered.

In addition to studies alluding to systemic alterations in acid-base balance, there have been a wide variety of observations over the years suggesting an association between abnormalities of local pH and the development of cancer. Some gastrointestinal tumors appear to be both preceded and accompanied by micro-environmental conditions in which the local acid-base equilibrium has been temporarily shifted in the alkaline direction. Deficiencies of gastric acid secretion are associated with carcinoma of the stomach and also with other tumors [3, 10]. In the more distal parts of the gastrointestinal tract, cancers have developed at sites at which local alkalinity may have been induced by an excessive elimination of cations [11, 12]. In the Plummer–Vinson syndrome, development of esophageal cancer may be prevented by the administration of iron and hydrochloric acid [13]; although it is not known if hydrochloric acid alone would have the same preventive effect.

The present study was undertaken using current methods of determining clinical acid-base status to determine whether a tendency to systemic alkalosis is present in patients with solid tumors.

MATERIAL AND METHODS

Patient selection

Arterial blood gas analyses (ABG) were performed on 18 normal volunteers and on

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126 patients, hospitalized between 1970 and 1975 at the Medical College of Georgia.

Group 1 consisted of 18 healthy volunteers—eleven females and seven males (ages 21–34, mean 26). These individuals were on no medication, with the exception of five women who were taking oral contraceptive preparations. In this group of volunteers we were unable to detect sex related variations in arterial blood gas values.

Group 2 consisted of 83 consecutive patients studied regarding ABG who were hospitalized with a variety of acute and chronic medical and surgical problems. This group was divided into three subgroups: A, B and C, according to their age on admission: 2A: range 19–34, mean 26; 2B: range 35–53, mean 46; 2C: range 54–77, mean 61. Subgroups A and C were of approximately the same mean age as Groups 1 and 3 respectively. No separation was made regarding sex, diagnosis, severity of the patient's disease process, or medications.

Group 3 consisted of 32 patients—30 males and two females (ages 45–74, mean 61) in whom a diagnosis of lung cancer was clinically suspected and subsequently confirmed. The cell types and frequency of these tumors were as follows: squamous 19, bronchogenic 11, alveolar 1 and clear cell 1. Two patients in this group (Nos. 15 and 18)* complained of chest pain. Three patients (Nos. 17, 20, 26) had severe obstructive ventilatory impairment.

Three patients (Nos. 6, 10, 12) had mild to moderate obstructive ventilatory impairment. One patient (No. 31) had severe restrictive ventilatory impairment. Two patients (Nos. 9 and 15) had mild restrictive ventilatory impairment. Three patients (Nos. 13, 18, 24) had mild to moderate obstructive and restrictive ventilatory impairment. One patient (No. 21) had lobar atelectasis. The predicted normal values for pulmonary function tests were calculated from standard nomograms [14].

Group 4 consisted of 11 hospitalized patients with histologically diagnosed solid tumors of different tissue origin: 7 female and 4 males (ages 25–73, mean 53). Nine of the eleven fell into the age range of Groups 2C and 3, (ie., 48–73). Hospitalization was at various times after the initial diagnosis (0–6 years, mean 2·3 years). The original sites of the tumors are given in the appendix. Only one patient (No. 9) had recently received a course of chemotherapy (vincristine and cyclophosphamide). None of the other patients were receiving drugs. Two patients (Nos. 6, 9) complained of mild to moderate intermittent pain during the time of the study. No other conditions known to affect acid base balance were detected in this group. All patients in Groups 3 and 4 were studied during a symptomatic period of their disease. None of the patients in the two cancer groups had clinical or laboratory evidence of abnormal liver or renal function, serum glucose values which were suggestive of diabetes mellitus, hypercalcemia, hypocalcemia, hyper- or hypokalemia. Cancer patients receiving diuretics, corticosteroids, antacids, or other drugs known to affect acid-base balance were excluded from the study. There was no evidence of clinical dehydration, a history of recent vomiting in any of these patients, or suggestion of abnormal hormonal production known to happen with some tumors.

Methods

The collection and analysis of arterial blood samples were performed identically in all groups. Samples were collected in 10 ml heparinized plastic syringes, immediately immersed in ice and transported to the blood gas laboratory. Analysis was performed within ½ hr after collection, using an Instrumentation Laboratories Blood Gas System, Model 213. The measured pH values were tabulated and corrected to a PCO_2 of 40 mm Hg using the Sigaard–Andersen nomogram [15, 16]. This "corrected" pH is designated as the "metabolic" (met.) pH. Hydrogen ion concentrations were calculated from the measured pH by standard methods. Calculated bicarbonate values are listed in Table 1.

Student's "*t*" test was used to determine the significance of differences of group means. Duncan's multiple range test was employed for non-parametric comparisons of groups.

RESULTS

Data from the arterial blood gas studies of Group 1, 2, 3 and 4 are summarized in Table 1. The *P* values listed for each variable in Table 1 represent the results of Student "*t*" test of the differences between the mean value of that group as compared to that for the normal volunteers (Group 1). Figure 1(a) graphically shows the distribution of measured arterial blood gas values between the normal volunteers and the two cancer groups. Figure 1(b) shows the distribution of measured arterial blood gas values between the two cancer groups and the inpatient population. It is worth noting that none of the 144 individuals studied had a

*See Appendix for individual values.

Table 1. *Normal volunteers—group* 1

No. Cases		Ages	pH	$PaCO_2$ mm Hg	HCO_3^- mEq/L	Met. (pH)	H^+ (nmole/L)
18							
	Range	21–34	7·36–7·45	31·7–40·2	21·0–25·5	7·36–7·42	35·5–43·6
	Mean	26	7·41	36·3	23·1	7·39	38·6
	S.D.		0·02	2·8	1·5	0·02	1·8
			Inpatient population—group 2 (Subgroups A, B and C)				
(A) 13							
	Range	19–34	7·41–7·48	29·1–37·4	20·0–24·0	7·38–7·43	33·1–38·9
	Mean	26	7·44	33·2	22·2	7·39	36·0
	S.D.		0·02	2·8	1·3	0·02	1·9
	*P**		+ <0·001	− <0·01	NS	NS	− <0·001
(B) 39							
	Range	35–53	7·36–7·54	28·1–48·7	18·6–30·5	7·33–7·50	28·8–43·6
	Mean	46	7·43	36·4	23·8	7·41	37·0
	S.D.		0·04	4·2	2·4	0·04	3·0
	*P**		+ <0·05	NS	NS	+ <0·05	NS
(C) 31							
	Range	54–77	7·36–7·50	27·5–48·3	18·4–29·8	7·36–7·47	31·6–43·6
	Mean	61	7·43	37·6	24·6	7·42	37·0
	S.D.		0·03	4·5	2·6	0·03	2·7
	*P**		+ <0·05	NS	+ <0·05	+ <0·01	− <0·05
			Lung cancer—group 3				
32							
	Range	45–74	7·40–7·57	27·5–45·0	19·9–34·8	7·36–7·51	26·9–39·8
	Mean	61	7·48	35·7	26·4	7·44	33·5
	S.D.		0·03	4·1	2·9	0·03	2·5
	*P**		+ <0·001	NS	+ <0·001	+ <0·001	− <0·001
			Other solid tumors—group 4				
11							
	Range	25–73	7·45–7·53	32·0–43·0	23·9–33·7	7·42–7·52	29·5–35·5
	Mean	53	7·48	36·5	27·6	7·46	32·9
	S.D.		0·03	3·4	3·0	0·04	2·1
	*P**		+ <0·001	NS	+ <0·001	+ <0·001	− <0·001

Groups 1, 2, 3 and 4: Arterial blood gases (ABG) data.
*P**—Significance of the difference from the mean of Group 1 (Student's "*t*" test).

Table 2.

Groups	1	2A	2B	2C	3	4
Status	Normal	In-patient subgroups			Lung cancer	Other tumors
Number	18	13	39	31	32	11
pH ($\mu \pm$ S.D.)	7·41 ± 0·02	7·44 ± 0·02	7·43 ± 0·04	7·43 ± 0·03	7·48 ± 0·03	7·48 ± 0·03
		N.S.		$P < 0·001$		
		N.S.		$P < 0·001$		
Met. pH ($\mu \pm$ S.D.)	7·39 ± 0·02	7·39 ± 0·02	7·41 ± 0·04	7·42 ± 0·03	7·44 ± 0·03	7·46 ± 0·04
		N.S.		$P < 0·01$		
		$P < 0·05$		$P < 0·01$		

Means, standard deviations and *P* values between the different groups according to the Student's "*t*" test.

Table 3. Duncan's Multiple Range Test. Means of groups that are not significantly different at the 0·01 level are underlined. The non-parametric comparison shows that both groups of cancer patients (3 and 4) are significantly different from the volunteers (1) and hospital patients of different ages (2A, 2B, and 2C)

	1	2A	2B	2C	3	4
pH	7·41	7·44	7·43	7·43	7·48	7·48
Metabolic pH	7·39	7·39	7·41	7·42	7·44	7·46

measured pH below 7·35. Table 2 shows the means, standard deviations and P values among the different groups. Table 3 shows the statistical differences at the 0·01 level according to the Duncan's Multiple Range Test. The measured arterial pH, (hydrogen ion concentration) and the corrected "metabolic" pH in the two groups of patients with solid tumors show a significant shift in the alkaline direction when compared to a control group of normal volunteers and to three subgroups of hospitalized patients with diverse diseases.

Although the differences between the pH values of the older consecutively hospitalized patients of Group 2C and the cancer patients of Groups 3 and 4 do not seem as impressive as those seen with the volunteers or the younger patients, there still is a clear separation between groups. Both pH and metabolic pH values are significantly higher in Groups 3 and 4 according to the Student "t" test ($P < 0·001$ and $P < 0·01$) and Duncan's multiple range test ($P < 0·01$) than in the older inpatient population (2C) (see Tables 2 and 3).

DISCUSSION

Our findings using modern techniques are in agreement with observations made by other investigators in decades past [4, 5, 9] and suggest that there is an unexplained tendency toward alkalosis in cancer patients with solid tumors which have not yet progressed to the terminal stage.

It is well known that pain, fear and anxiety may be accompanied by both acute and chronic hyperventilation with an associated respiratory alkalosis. Metabolic alkalosis in cancer patients has also been reported to be directly related to the severity of pain [17]. Neither of these situations seems to pertain to our study. No significant differences in arterial

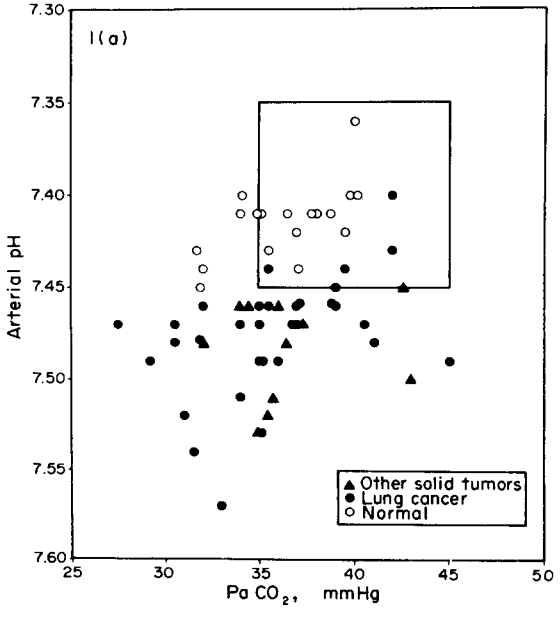

Fig. 1(a). Distribution of measured pH and $PaCO_2$ values in solid tumor groups (3 and 4) and normal volunteers (1). The smaller square indicates the normal range for arterial pH and $PaCO_2$.

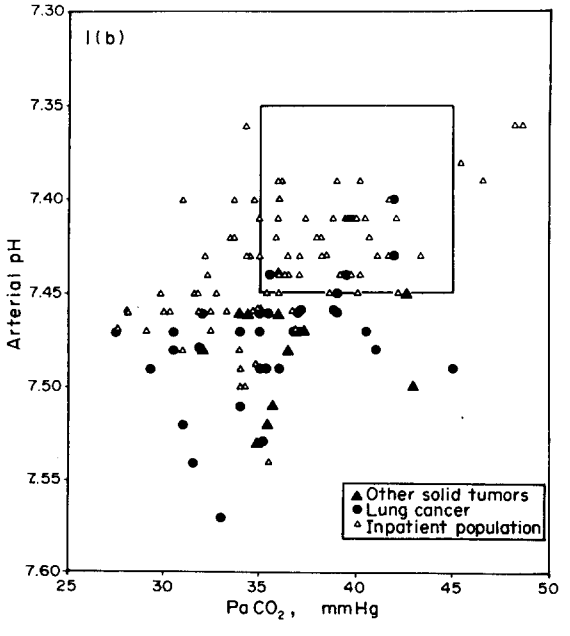

Fig. 1(b). Distribution of measured pH and $PaCO_2$ values in cancer groups (3 and 4) vs the consecutive inpatient population (group 2).

$PaCO_2$ exist between the cancer groups and the older subgroups of inpatients. Indeed a significantly lower mean value for arterial PCO_2 was found in the youngest subgroup of patients.

In addition to the increased rates of anaerobic and aerobic glycolysis and accumulation of lactic acid, a tendency toward decreased glucose levels in the venous effluent of tumors has been reported in association with cancer by some investigators [18–22]. These same three characteristics: an accumulation of lactic acid, increased glycolysis, and decreased glucose levels, can be produced experimentally by increasing the alkalinity of the blood [23–26]. Huckabee has shown in humans that hypoxia is not the only circumstance in which lactate production is increased [27]. He and others have documented that an elevation of pH, of *either* respiratory or metabolic origin accelerates the process of glycolysis and the formation of lactate [27–29]. This alkalosis mediated acceleration of glycolysis has been shown to be induced principally through stimulation of the phosphofructokinase enzyme reaction [29, 30]. Therefore, in many ways alkalosis, independent of the cause, seems to act on the physiology of the organism in a manner similar to hypoxia. It can thus be concluded that many of the abnormal metabolic characteristics of malignant cells and tissues can be induced in normal cells by stimulus of a higher than normal pH.

Reding and Slosse in 1929 [5, 9] reported that a tendency toward alkalinity in the blood exists at the time of the first clinical manifestations of cancer. Our findings, particularly in the lung cancer patients, Group 3, lend support to their contention.

The basis for the association between alkalosis and cancer from the time of its initial clinical manifestations has not been defined, nor obviously has the question of cause and effect been resolved. However, a possible primary role of acidosis in stimulating the immune response has been advanced [31, 32], and a recent report has described tumor inhibition following the induction of systemic acidosis in different animal malignancies [8]. Therefore, the possibility that local and systemic alterations in acid-base balance in the alkalotic direction play an important role in the genesis and/or propagation of cancer remains not only a viable but a vital issue at the present time.

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APPENDIX

Table A1. *Individual data—group* 1

Case	Age–Sex	pH	$PaCO_2$ (mm Hg)	HCO_3^- (mEq/L)	Met. (pH)	H^+ (nmole/L)
1	28 M	7·41	38·0	24·0	7·40	38·9
2	30 F	7·41	35·1	22·1	7·37	38·9
3	34 M	7·40	39·8	24·5	7·40	39·8
4	28 M	7·41	38·7	24·3	7·40	38·9
5	23 F	7·44	32·0	21·5	7·38	36·3
6	21 F	7·42	37·0	24·0	7·40	38·0
7	28 F	7·41	34·0	21·4	7·36	38·9
8	27 F	7·41	34·9	22·0	7·38	38·9
9	22 F	7·40	34·1	21·0	7·36	39·8
10	22 F	7·41	36·5	22·7	7·39	38·9
11	24 F	7·36	40·0	22·3	7·36	43·6
12	26 F	7·45	31·9	22·0	7·39	35·5
13	31 F	7·43	31·7	21·0	7·37	37·1
14	27 M	7·42	39·5	25·5	7·42	38·0
15	28 M	7·44	37·1	25·0	7·41	36·3
16	23 M	7·40	40·2	24·8	7·40	39·8
17	21 F	7·43	35·5	23·4	7·40	37·1
18	28 M	7·41	37·8	23·8	7·39	38·9

Individual data—group 3

Case	Age–Sex	pH	$PaCO_2$ (mm Hg)	HCO_3^- (mEq/L)	Met. (pH)	H^+ (nmole/L)
1	63 M	7·46	39·0	27·6	7·46	34·7
2	63 M	7·46	37·0	26·4	7·44	34·7
3	68 M	7·48	41·0	30·6	7·49	33·1
4	70 M	7·47	34·0	24·8	7·42	33·9
5	58 M	7·54	31·5	27·2	7·47	28·8
6	55 M	7·57	33·0	30·6	7·51	26·9
7	68 M	7·49	45·0	34·8	7·51	32·4
8	64 M	7·49	35·0	26·8	7·45	32·4
9	62 M	7·44	35·5	24·2	7·41	36·3
10	63 M	7·46	32·0	22·8	7·40	34·7
11	66 M	7·53	35·0	29·5	7·49	29·1
12	63 M	7·44	39·5	26·8	7·44	36·3
13	61 M	7·48	30·5	22·4	7·40	33·1
14	64 M	7·49	29·2	22·3	7·40	32·4
15	45 M	7·52	31·0	25·5	7·45	30·2
16	56 M	7·45	39·0	27·0	7·45	35·5
17	62 M	7·47	40·5	29·2	7·48	33·9
18	59 M	7·43	42·0	28·8	7·45	37·1
19	62 F	7·46	35·0	24·8	7·42	34·7
20	62 F	7·47	37·0	27·0	7·45	33·9
21	67 M	7·40	42·0	25·8	7·42	39·8
22	63 M	7·47	35·0	25·4	7·43	33·9
23	74 M	7·47	30·5	22·2	7·39	33·9
24	57 M	7·49	36·0	27·4	7·46	32·4
25	62 M	7·49	35·0	27·0	7·46	32·4
26	48 M	7·47	37·0	27·0	7·45	33·9
27	65 M	7·46	27·5	19·9	7·36	33·9
28	55 M	7·46	35·5	25·7	7·43	34·7
29	69 M	7·51	34·0	27·3	7·46	30·9
30	62 M	7·48	32·0	24·0	7·42	33·1
31	63 M	7·46	39·0	27·8	7·46	34·7
32	63 M	7·46	37·0	26·5	7·44	34·7

Individual data—group 4

Case	Age–Sex	Origin	pH	$PaCO_2$ (mm Hg)	HCO_3^- (mEq/L)	Met. (pH)	H^+ (nmole/L)
1	48 F	Cervix	7·48	32·0	23·9	7·42	33·1
2	73 F	Sigmoid	7·45	42·6	29·4	7·47	35·5
3	63 F	Sigmoid	7·48	36·5	27·5	7·46	33·1
4	25 M	Rectum	7·46	34·5	24·3	7·42	34·7
5	61 F	Rectum	7·50	43·0	33·7	7·52	31·6
6	51 F	Breast	7·46	36·0	25·6	7·43	34·7
7	58 F	Breast	7·52	35·5	28·9	7·48	30·2
8	58 F	Breast	7·46	34·0	24·2	7·42	34·7
9	42 M	Testicle (seminoma)	7·53	35·0	29·5	7·49	29·5
10	60 M	Thyroid (follicular)	7·47	37·3	27·5	7·46	33·9
11	49 M	Neurofibrosarcoma	7·51	35·7	28·7	7·47	30·9

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Insulin-induced Growth Hormone Response in Patients with Uterus Carcinoma. I. Endometrial Carcinoma*

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Abstract—*The insulin-induced GH responses were studied in 37 patients with endometrial carcinoma and 11 healthy obese women. Mean GH concentrations were significantly lower in the cancer patients. Quarter of patients showed abnormally low pituitary GH release. The GH responses in obese patients with endometrial carcinoma were significantly lower than those in thin.*

INTRODUCTION

GROWTH hormone has been shown to result in tumor development in laboratory animals [1, 2]; and there has also been some evidence that tumors develop more frequently in patients with acromegaly [3]. Benjamin found in patients with endometrial cancer increased GH secretion after insulin administration as well as paradoxical rises in its concentration following a glucose load [4, 5]. This paper represents the studies on disturbances in hypothalamo-pituitary axis regulation which are conducted in patients with endometrial carcinoma.

MATERIAL AND METHODS

Thirty-seven patients with endometrial cancer (e.c.) and 11 healthy obese women (h.o.w.) were studied. All patients had histologically proven carcinoma and were in good general condition. Patients with clinical diabetes were excluded.

In order to make our conclusions more precise, patients were divided due to stage of disease and weight. Out of patients with e.c 28 were in stage I and 9 in more advanced stages.

"Desirable" body weight was obtained from the Tables of Metropolitan Life Insurance Company [6]. For a given height the mean of the range for "medium frame" was considered 100% and the criterion of obesity was an actual weight greater than 115%. Twenty-three patients with e.c. were obese. The results of insulin-induced GH responses in obese patients with e.c. were compared to those in h.o.w. Because of J. Roth report that there is no difference in GH responsiveness to stimulation tests between the obese and thin [7], we considered our group of h.o.w. as a control group to thin patients with e.c. as well.

After overnight fasting, patients and control women each received intravenously 0·1 units of regular insulin/kg b.w. Blood samples for glucose and GH concentrations were obtained prior to and at the 30, 45, 60, 90, 120 and 180 min after the insulin injection. All patients and controls experienced at least a 50% fall in serum glucose concentrations with concomitant symptoms of neuroglycopaemia. Serum GH concentrations were measured by means of CEA-IRE-Sorin kit, and plasma glucose levels by the method of Asatoor–King [8].

Student-*t* test was used for statistical analysis.

RESULTS

Serum GH concentrations after the insulin stimulation are shown in Table 1 and in Fig. 1. Mean serum GH level rose from 1·1 ± 0·26 ng/ml (mean ± S.E.) to 14·4 ± 2·90 ng/ml in whole group of patients with e.c., and

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Table 1. *Mean serum growth hormone concentrations following the insulin stimulation*

Time (min)		0	30	45	60	90	120	180
				ng/ml				
				endometrial carcinoma (e.c.)				
Whole group (n = 37)	Mean	1·1	2·8	8·0	14·4	11·0	6·4	2·9
	± S.E.	0·26	0·84	1·76	2·09	1·91	1·56	0·66
Thin (n = 14)	Mean	1·2	4·7	11·9	19·9	15·1	7·8	3·2
	± S.E.	0·35	2·03	3·46	3·55	2·74	1·96	1·14
Obese (n = 23)	Mean	1·0	1·7	5·2	10·9	8·4	5·5	2·6
	± S.E.	0·37	0·56	1·34	2·32	2·49	2·24	0·82
				healthy obese women (h.o.w.)				
(n = 11)	Mean	0·9	9·5	21·0	32·4	29·1	14·2	5·9
	± S.E.	0·34	3·48	4·95	5·33	6·51	3·80	1·45
	p e.c./ h.o.w.	N.S.	<0·01	<0·01	<0·001	<0·001	<0·05	<0·05
	p Thin/ Obese	N.S.	<0·001	<0·001	<0·001	<0·001	<0·01	N.S.

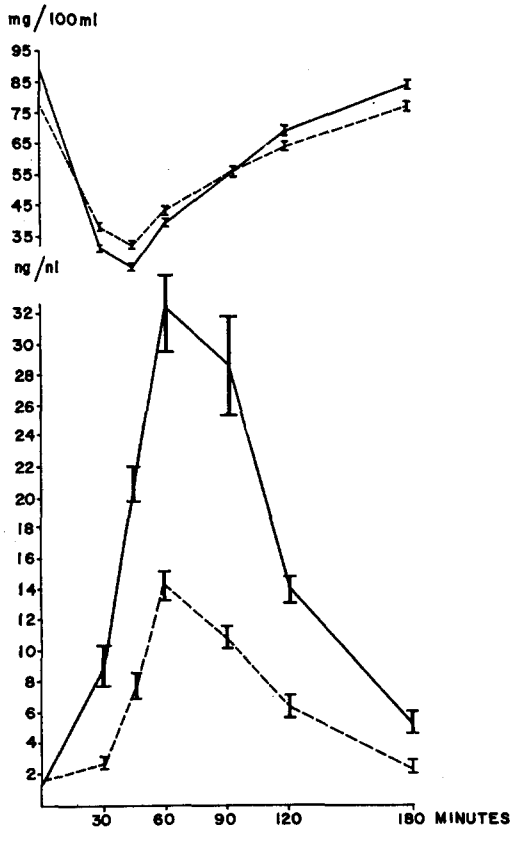

Fig. 1. *Mean serum GH and glucose concentration following the insulin stimulation in patients with endometrial carcinoma* (----) *and in control group* (——).

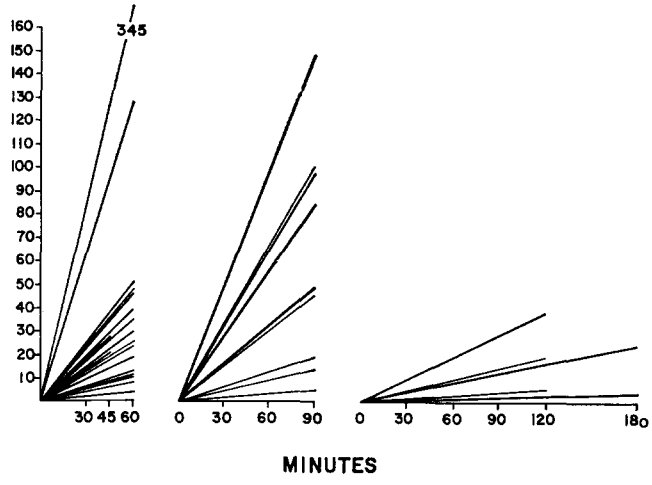

Fig. 2. *Multiplicity of maximum GH increments plotted against time in patients with endometrial carcinoma.*

from 0·9 ± 0·34 ng/ml to 32·4 ± 5·33 ng/ml in the controls. Mean GH concentrations following the insulin stimulation were significantly lower in patients with e.c. than in the controls. Nine patients (25% of total) showed less than 5 ng/ml increment of GH concentration; these patients were obese.

When responses to insulin stimulation were expressed as maximum multiplicity over the basal value of GH, 8 patients showed values below 10. Nine patients showed rise in GH concentrations at the 90th min; 5 of them were obese. Five patients showed rise in GH concentrations only after 120 min, and 3 did not any; all of them but one were obese (Fig. 2). Seventeen patients had the peak responses at or after the 90th min while only 3 out of 11 h.o.w. showed the peak at the 90th min and 2 had the value of multiplicity of increment less than 10 (Fig. 3).

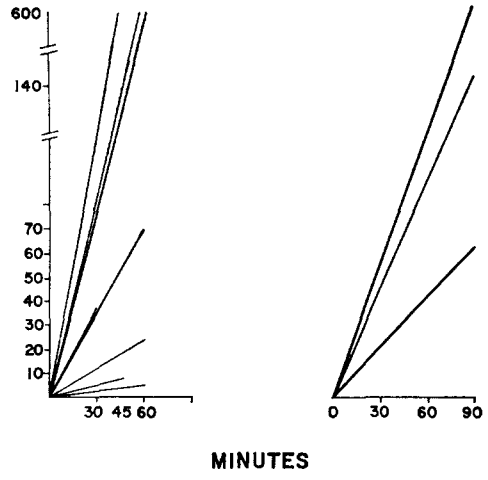

Fig. 3. Multiplicity of maximum GH increments plotted against time in control group.

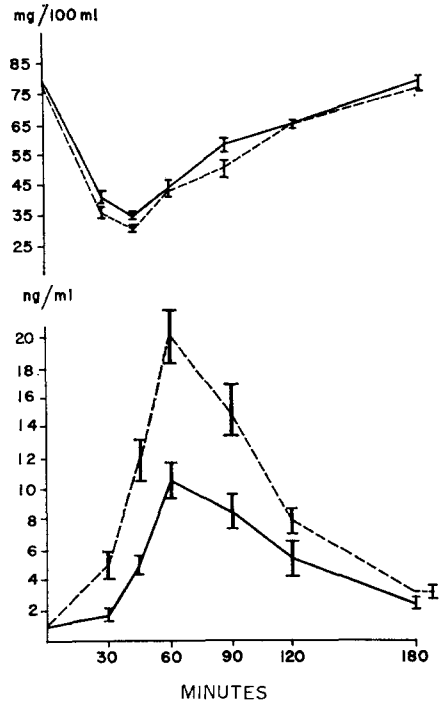

Fig. 4. Mean serum GH and glucose concentration following the insulin stimulation in thin (----) and obese (———) patients with endometrial carcinoma.

The GH responses to hypoglycemia were significantly lower in obese patients than in the thin (Table 1 and Fig. 4). We did not find any influence of stage of disease on the results.

DISCUSSION

Many authors have been unable to document any significant difference in GH release in men which could be related to age or sex [9, 10]. Johansen has given evidence that there is a decrease in the metabolic clearance rate of GH in diabetics, rather than increase in secretion [11]. Obesity decreases GH responsiveness to stimulation tests [12]; but some reports notify that there is no difference between the obese and thin [7]. We have found that survival rate after the cobalt-therapy was higher in obese patients with e.c. than in the thin ones. Does this fact depend upon the different hormonal environment?

We found serum GH concentrations after insulin injection significantly lower in patients with e.c. than in h.o.w.; this observation is in variance to that of Benjamin [4]. Eight out of 37 patients with e.c. reached a peak of GH response at the 120th min or later, and 5 of them had abnormally low GH responses (less than 5 ng/ml). It means that 13% of patients had GH deficiency and other 9% showed delayed pituitary release. All of them but one were obese. It might be useful to measure not only serum GH concentrations but also the amount or capacity of tissue receptors for this hormone. It is conceivable that despite of normal GH level the capacity of GH receptors may be increased as in some cases of breast cancer [13]. At the present time the role played by GH in endometrial carcinoma remains unclear.

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Prognosis in Inoperable Stage III Carcinoma of the Breast

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Abstract—*One hundred and eighty-four patients with inoperable Stage III breast cancer presenting to the Guy's Hospital Breast Unit between* 1961–1973 *were treated initially by radiotherapy alone. The response rate was* 60% *but duration of response and survival were short. Seventy-two per cent of patients did not have prolonged control of local disease and* 63% *developed distant metastases.*

The association between certain prognostic variables and response to radiotherapy, subsequent development of metastases and survival was analysed statistically. The duration of response to radiotherapy showed no significant associations with any prognostic variable. Subsequent distant metastases occurred less often in patients responding to radiotherapy, having a subsequent mastectomy or if the duration of symptoms before presentation was long, but more frequently if the primary tumours were diffuse. Survival was shorter in patients who were early postmenopausal, had a short duration of symptoms or had diffuse primary tumours. Improved survival was associated with a good response to radiotherapy and, unexpectedly, with deep fixation of the primary tumour. Prognosis was not significantly associated with size of primary tumour or involvement of skin or lymph nodes. The effectiveness of combining variables in predicting prognosis is described.

A further group of 30 *patients, unsuitable for radiotherapy were treated primarily by additive endocrine therapy and had a median survival of* 14 *months.*

The clinical course of Stage III breast cancer is variable, there being two extremes: a slowly-growing, non-metastasising form and a more common, rapidly-growing, metastasising form. For prognosis to be improved, systemic therapy as part of the primary management of this disease may be necessary. Prognostic variables should be considered in the design and assessment of future clinical trials.

INTRODUCTION

THE FACTORS contributing to the inoperability of locally advanced breast carcinoma (Stage III) are large size of the primary tumour, involvement of the overlying skin, satellite skin nodules, peau d'orange, attachment to deep structures, matting or fixation of axillary lymph nodes and involvement of supraclavicular lymph nodes. Although these factors may not necessarily render operative removal technically impossible, the results of surgery for this type of disease are poor. There is a high incidence of relapse and survival is short [1], and the preferred conventional treatment for this type of breast cancer is radiotherapy. This usually gives satisfactory control of local disease in adequate dosage [2], but survival is still poor (median less than three years), and most patients relapse with distant metastases [3]. Seventeen per cent of patients with localised breast cancer presenting to the Guy's Hospital Breast Unit have inoperable disease.

In this paper we describe the clinical course of patients with locally advanced, inoperable breast cancer, classified as Stage III by the TNM classification [4], analyse factors which affect prognosis and speculate on the role of primary systemic treatment in this condition.

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MATERIAL AND METHODS

Radiotherapy

One hundred and eighty-four patients with Stage III breast cancer presenting at the Guy's

Hospital Breast Unit during the 13-year period 1961–1973 who were treated primarily by radiotherapy have been studied. Stage III is defined using the TNM classification [4] as: either $T_{3,4}$ any N, M_0 or any T, $N_{2,3}$, M_0. Included are 13 patients with T_{3a}, $N_{0,1}$, M_0 tumours who would normally have been treated by radical mastectomy at this Unit, but, because they were unfit for surgery, were treated primarily by radiotherapy. Each patient received 3600–4000 rad in 3–3½ weeks by tangential fields to the chest wall by a 4 MeV linear accelerator with skin bolus (15 fractions) followed by 3000 rad in two weeks to the ipsilateral local gland fields at 250 kV.

Before radiotherapy, all lesions in the breast and regional lymph glands were measured. These were used as a baseline on which to assess the response to radiotherapy. Full physical examination and chest and skeletal radiography was done to exclude the presence of detectable distant metastases to confirm the M_0 status.

The therapeutic response to radiotherapy was assessed as follows:

1. Complete regression (CR): complete disappearance of all visible and palpable disease.
2. Partial regression (PR): a decrease of 50% or more in the sum of the products of the largest perpendicular axes of the individual lesions.
3. No change (NC): less than 50% decrease or a less than 25% increase in the sum of the products of the largest perpendicular axes of the individual lesions.
4. Progressive disease (PD): a greater than 25% increase in the sum of the products of the largest perpendicular axes of the individual lesions, and/or the appearance of new local lesions or distant metastases.

The duration of response (for CR, PR and NC) is the time from the date of commencement of radiotherapy to the date of documentation of progressive disease. When progression of disease occurred after radiotherapy, patients received subsequent treatment as appropriate, including palliative simple mastectomy, further radiotherapy, oophorectomy, androgens, oestrogens, hypophysectomy, corticosteroids and cytotoxic chemotherapy. Sites of subsequent distant metastases were documented and the survival of patients from the time of diagnosis of Stage III breast cancer was recorded. Minimum follow-up for this series is 28 months.

Certain factors ("prognostic variables") were studied to determine whether significant associations existed between them and the therapeutic response to radiotherapy, development of distant metastases and survival as follows: (a) The year of first presentation, diagnosis and primary treatment (always the same calendar year); (b) Menopausal status at diagnosis (premenopausal, including those having a menstrual period in the previous year; postmenopausal 1–5 years, 6–10 years, >10 years); (c) Duration of symptoms; (d) Size of primary tumour (largest dimension); (e) Skin involvement (fungation, infiltration of skin overlying primary, satellite skin nodules over the breast, peau d'orange); (f) Fixation to deep structures; (g) Node involvement ($N_{0,1,2,3}$ according to the TNM classification [4]. The association between the response to radiotherapy and subsequent palliative mastectomy (in 25 patients) with the eventual development of distant metastases and survival was also analysed.

Statistical analyses

The aim of the statistical analyses has been to assess the extent to which the "response variables" (response to radiotherapy, development of metastases and survival) were associated with various factors or "prognostic variables". The method of analysis depended on the nature of the variable concerned. The development of metastases at any site was taken as a binary variable and analysed by logistic regression. In some analyses of response to radiotherapy this was also treated as a binary variable by counting CR or PR as a positive response; these analyses also used logistic regression. Other analyses of response to radiotherapy retained the four categories and used an extension of logistic regression for polytomous variables. Survival data was of the usual "censored" type, with some complete measurements of survival for patients who had died, and other incomplete observations for patients still alive. Cox's [5] method of analysis was used to assess the association with prognostic variables. The survival experience of the whole group, and of various subgroups, was analysed by standard life-table methods.

In the various analyses relating response variables to prognostic variables, various alternative combinations of the latter were used. Also, alternative methods of specifying variables were used: for instance, the year of the study was used both as a quantitative variable to see whether any smooth trend was apparent, and as a qualititative variable by using seven

time periods and distinguishing between them by dummy variables.

In statements of the results of significance tests $\chi^2_{(f)}$ means χ^2 on f degrees of freedom.

Endocrine therapy

A small group of 30 patients with Stage III carcinoma of the breast who presented during the same period, were unsuitable for radiotherapy, either because of old age and infirmity or because the disease was so extensive as to render local treatment technically impracticable. They were treated initially by additive hormones with either oestrogens (23 patients) or androgens (7 patients). The survival of these patients has also been analysed for comparison with those treated by radiotherapy, but, because of the small number, prognostic variables have not been studied.

RESULTS

Patients treated by radiotherapy

Table 1 shows the principal characteristics of these patients, separately for each of three time periods of 4–5 years. The distributions of the various characteristics did not change greatly during the 13 years, the most noteworthy change being perhaps a lengthening of the duration of symptoms.

Table 1. *Characteristics of the patients with Stage III breast cancer treated by radiotherapy (Percentages between parentheses)*

	1961–1965	1966–1969	1970–1973	Total
Number of patients	41	67	76	184
Age (years)				
30	3 (7)	0 (0)	4 (5)	7 (4)
40	6 (15)	13 (19)	15 (20)	34 (18)
50	15 (37)	30 (45)	23 (30)	68 (37)
60	13 (32)	17 (25)	22 (29)	52 (28)
70	4 (10)	5 (7)	11 (14)	20 (11)
80	0 (0)	2 (3)	1 (1)	3 (2)
Menopausal status				
Pre- and < 1 yr postmenopausal	10 (24)	19 (28)	22 (29)	51 (28)
Postmenopausal 1–5 years	5 (12)	10 (15)	13 (17)	28 (15)
Postmenopausal 6–10 years	12 (29)	13 (19)	8 (11)	33 (18)
Postmenopausal > 10 years	14 (34)	25 (37)	33 (43)	72 (39)
Duration of symptoms (months)				
0	39 (95)	61 (91)	64 (84)	164 (89)
24	1 (2)	1 (1)	4 (5)	6 (3)
48	0 (0)	3 (4)	5 (7)	8 (4)
72	0 (0)	2 (3)	3 (4)	5 (3)
Unknown	1 (2)	0 (0)	0 (0)	1 (1)
Largest diameter (cm)				
0	6 (15)	10 (15)	17 (22)	33 (18)
5	20 (49)	39 (58)	46 (61)	105 (57)
10	9 (22)	12 (18)	11 (14)	32 (18)
Diffuse	6 (15)	5 (7)	2 (3)	13 (7)
Unknown	0 (0)	1 (1)	0 (0)	1 (1)
Skin				
No attachment or involvement	1 (1)	4 (6)	4 (5)	9 (5)
Attachment only—no involvement	13 (32)	17 (25)	21 (28)	51 (28)
Involvement	27 (66)	46 (69)	51 (67)	124 (67)
Deep fixation				
–	35 (85)	43 (64)	51 (67)	129 (70)
+	6 (15)	24 (36)	25 (33)	55 (30)
N-staging				
0	12 (29)	16 (24)	23 (30)	51 (28)
1	15 (37)	28 (42)	31 (41)	74 (40)
2	8 (20)	11 (16)	16 (21)	35 (19)
3	6 (15)	12 (18)	6 (8)	24 (13)

Response to radiotherapy

Table 2 shows the number and duration of responses for patients in each of the response categories; the poorer responses being associated with lower durations. Of the 24 patients with PD, 17 failed to have regression of disease locally; the remaining 7 patients developing distant metastases before the completion of radiotherapy. A further 115 patients (in the CR, PR and NC categories) subsequently relapsed in the irradiated field. Thus a total of 132 patients (72%) failed to have prolonged control of local disease.

Table 2. Response to radiotherapy

Response	Number of patients (%)	Duration (months) Mean	Range
CR	34 (19)	17.26	3–56
PR	73 (41)	12.48	2–43
NC	49 (27)	7.59	2–18
PD	24 (13)	0.00	0–0
	180 (100)		
Incomplete information	4		
Total	184		

Table 4. Subsequent distant metastases: relation to response to radiotherapy

Response to radiotherapy	Number of patients With metastases	Without metastases
CR+PR	64	44
NC+PD	56	17
Unknown	1	2

$\chi^2_{(1)} = 5.2$; $P = 0.02$.

Subsequent distant metastases in patients treated by radiotherapy

Table 3 shows the numbers and proportions of patients who developed subsequent metastases at various sites.

Table 4 shows that the incidence of subsequent metastases at any site is lower for patients showing a response to radiotherapy than for those not responding ($\chi^2_{(1)} = 5.2$; $P = 0.02$) and that it is lower ($\chi^2_{(1)} = 3.2$; $P = 0.07$), although not significantly so, for those who had a palliative mastectomy than for those who did not (Table 5).

A logistic regression shows that the incidence of subsequent metastasis decreases with the duration of symptoms, the association being highly significant ($\chi^2_{(1)} = 9.1$; $P = 0.003$).

Table 3. Incidence of metastases at distant sites developing in patients treated by radiotherapy

	Bone	Pleura	Lung	Liver	Peritoneum (ascites)	Brain	Other	Any sites
Number	61	43	27	35	13	9	3	121
%	33	23	15	19	7	5	2	63

Analyses of response, either as a binary variable or as a 4-level variable, showed no significant overall association with prognostic variables. In the latter analysis, though, some of the individual regression coefficients reached significance, suggesting that more than 10 years postmenopausal, and a diffuse tumour were each detrimental.

The proportion of responses showed no clear trend during the 13-year period, but there were curious fluctuations from year to year; for example 13 responses in 27 patients in 1969, 16 in 16 in 1970 and 18 in 24 in 1971.

The duration of response, analysed by Cox's method, showed no significant associations with any prognostic variable.

Table 6 illustrates this association. When several prognostic variables were introduced simultaneously the effect of duration of symptoms remained significant ($\chi^2_{(1)} = 6.3$; $P = 0.01$), and a diffuse tumour was associated with a higher risk of subsequent metastases ($\chi^2_{(1)} = 5.2$; $P = 0.02$); the effect of year was not quite significant ($P = 0.08$), but suggested a reduction in the chance of metastases during the 13-year period. However, this may just reflect the shorter follow-up periods for patients presenting in more recent years.

Survival after radiotherapy

The survival pattern of the whole series, calculated by life-table methods, is shown in

Table 5. *Subsequent distant metastases: relation to palliative simple mastectomy after radiotherapy*

	Number of patients	
	With metastases	Without metastases
Palliative mastectomy	12	13
No mastectomy	109	50

$\chi^2_{(1)} = 3.2$; $P = 0.07$.

Table 6. *Subsequent distant metastases: relation to duration of symptoms*

	Number of patients	
	With metastases	Without metastases
Duration of symptoms		
Less than 6 months	80	39
6 or more months	41	33
Unknown	0	1

$\chi^2_{(1)} = 9.1$; $P = 0.003$.

Fig. 1. The median survival time after radiotherapy is estimated as 25 months. Twenty-eight per cent of patients survived more than 3 years, and 13% more than 5 years.

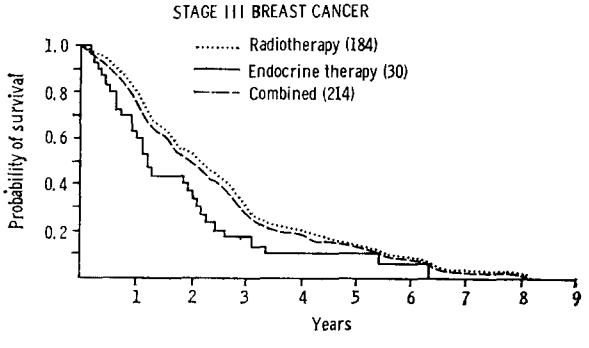

Fig. 1. *Survival curves for* 184 *patients treated by radiotherapy,* 30 *patients treated by endocrine therapy and the combined group* (214 *patients*).

The results of a Cox analysis, using five prognostic variables, are summarized in Table 7. The regression coefficients, shown in that table with their standard errors, measure the extent to which the natural logarithm of the force of mortality (the instantaneous death rate amongst those who have survived any given time) is affected by changes of one unit in each of the prognostic variables. The variables shown in Table 7 were chosen after a series of preliminary analyses using each of these factors, and several others, individually; those shown in Table 7 were the factors which, when used alone, were significantly associated with survival.

Table 7. *Analysis of survival by Cox's method*

Prognostic variable	Regression coefficient ± standard error
Year of diagnosis	−0.095 ± 0.027
Menopausal status (if 1–5 yrs postmenopausal)	0.41 ± 0·21
Log. duration of symptoms	−0.103 ± 0.074
Tumour (if diffuse)	0.44 ± 0.32
Deep fixation	−0.51 ± 0.18

The joint effect of all the variables shown in Table 7 is highly significant ($\chi^2_{(5)} = 35.7$; $P < 0.001$). Each variable shows a separate effect greater than its standard error, those for year and deep fixation being highly significant. The mortality rate declined during the 13-year period in a way that cannot be attributed to changes in other known prognostic variables; deep fixation is associated with *better* prognosis. Of the other three, less significant, variables, women 1–5 years postmenopausal had a rather shorter survival, and survival was longer for patients with a longer duration of symptoms and shorter for those with diffuse tumours.

Subsequent analyses, not reported in detail here, showed that the use of further information about the size of tumour, nodal grading and skin fixation made no appreciable difference to the prognosis, as judged by the χ^2 value. The addition of response to radiotherapy and of palliative mastectomy each made a highly significant improvement in prognostic effectiveness (survival being longer for patients showing positive response and for those having palliative mastectomy), but of course these are two variables which are not measurable at the start of treatment.

To confirm the effectiveness of prognosis based on the variables listed in Table 7, the patients were divided into five subgroups of approximately equal size, according to the value of the expression which predicts the mortality rate by appropriate weighting of these variables. The life-table for each subgroup was then calculated. The results are

shown in Fig. 2. There is a fairly clear gradation between the results, with groups 2 and 3 less clearly distinguished than the others. The contrast between the extreme groups corresponds to an approximate doubling of survival between groups 5 and 1.

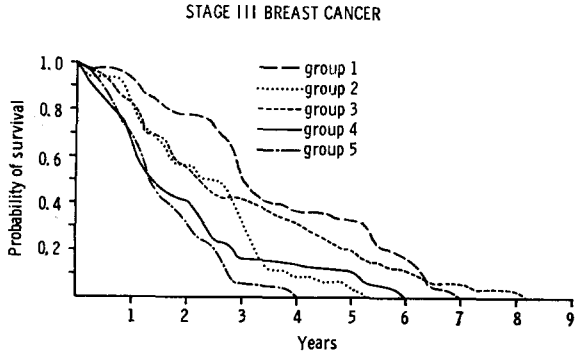

Fig. 2. Survival curves for five subgroups of patients treated by radiotherapy, distinguished by prognostic factors. Group 1 has the lowest risk, group 5 the highest.

This division into groups is influenced considerably by the effect of the "year" variable, which implies that prognosis is affected by unidentified factors which change gradually over the whole period.

Survival after endocrine therapy

The survival pattern after endocrine therapy calculated by life-table analysis is shown in Fig. 1; survival is on the whole worse than for the patients treated by radiotherapy.

The 23 patients treated by oestrogens were elderly (median age 71 years; range 56–90); 18 (78%) were over 70 and 10 (43%) were over 80, only one was under 60. The median survival for this group was 14 months, but 5 patients (22%) survived 3 years and 3 of them (13%) 5 years.

The 7 patients treated by androgens (median age 54 years, range 47–79) also had a median survival of 14 months, but none survived more than 29 months.

Figure 1 also shows the life-table survival for the whole group of 214 patients treated by either radiotherapy or endocrine therapy.

DISCUSSION

A proportion of patients with localized breast cancer (17% of those referred to this Unit) have inoperable disease, the preferred treatment being radiotherapy. Although the initial response rate to this form of treatment is high, more than half the patients have progressive disease within 18 months. In this study 72% of patients had progressive local disease and 63% had developed evidence of distant metastases within the minimum follow-up period of 28 months; survival was short (median 25 months). The radiotherapy in the doses used here, can, therefore, only be considered palliative, although similar results were reported by Zucali *et al.* [3] using considerably higher radiation doses. There was no definite association between the response to radiotherapy and any of the prognostic variables identifiable at the time of presentation. Distant metastases occurring after radiotherapy were less in patients having a good response (CR + PR) to radiotherapy (Table 4), a subsequent palliative mastectomy because local primary disease was not controlled (Table 5), and if the duration of symptoms before presentation had been long (Table 6). Diffuse primary tumours were associated with a high incidence of subsequent distant metastases.

Significant correlations were found between survival and certain of the prognostic variables. In particular, survival was poor in patients whose last menstrual period had occurred 1–5 years before presentation, had a short duration of symptoms before presentation (less than six months), and with diffuse primary tumours. Unexpectedly, deep fixation of the primary tumour was associated with improved survival and the explanation for this is obscure. Response to radiotherapy, (CR or PR) was also associated with better survival. The mortality rate throughout the 13-year period of the study showed a steady decline which cannot be attributed to changes in the other known prognostic variables. This implies that other unknown changes occurred during this period and cautions against the use of historical controls in therapeutic trials. The effectiveness of combining these variables has been clearly illustrated (Fig. 2). Although large size of primary tumour and extensive regional nodal involvement have been shown to be adversely associated with survival in other studies [3, 6], this was not found in this study, nor was there any significant correlation between skin involvement and prognosis.

This study shows that the clinical course of Stage III breast cancer is highly variable. At one extreme there is a slow growing, non-metastasizing form in which patients have a long duration of symptoms before presentation; at the other extreme is a more common, rapidly growing metastasizing form in which patients have a short history.

The small group of patients in this series treated primarily by additive hormone therapy

had a poorer survival than those treated by radiotherapy. This was to be expected as these patients were selected for systemic treatment because either their disease was too extensive to be treated locally or they were old and infirm. However, it is clear that the majority of patients with Stage III breast cancer eventually develop distant metastases which are present in an occult form at the time of presentation and that more radical local therapy does not improve the prognosis. It is, therefore, pertinent to consider the possibility of incorporating systemic therapy into the primary management of Stage III disease. Breast cancer is the one common tumour which is frequently sensitive to cytotoxic chemotherapy and study of the use of this form of treatment in Stage III disease has begun [7]. The outlook in Stage III breast cancer may well improve by combining local and systemic therapies, but the true place of any treatment (surgery, radiotherapy, chemotherapy, endocrine therapy, immunotherapy) will be known only after critical evaluation by controlled clinical trials. Information gained from the prognostic variables discussed here should be considered in both the design and assessment of such trials.

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Effect of Progesterone and Estrogen on DNA Synthesis of Pregnancy-Dependent Mammary Tumors in GR/A Mice*

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Abstract—*The effects of progesterone and estrogen on the growth and DNA synthesis of pregnancy dependent mammary tumors of GR/A mice were studied. One group of mice with palpable mammary tumors received daily subcutaneous injections of* 3*mg progesterone plus* 0.5*μg estradiol benzoate in combination during days* 14 *and* 19 *of pregnancy and ovariectomized bilaterally on day* 15. *Another group of mice was given only the unilateral ovariectomy on day* 15. *These treatments resulted in the increase in mammary tumor size and the prevention of decrease in DNA synthesis (estimated by the* in vivo *incorporation of* (^3H) *thymidine) of normal and neoplastic mammary glands on day* 19 *of pregnancy. On the other hand, mammary tumor size changed little and DNA synthesis of either gland was significantly decreased in ovariectomized mice receiving* 2*mg progesterone alone. Three mg progesterone increased DNA synthesis of mammary tumors, but not of normal glands as compared to* 2*mg progesterone. These results indicate that both progesterone and estrogen are essential for the growth of pregnancy-dependent mammary tumors and that there are some differences in responsiveness to progesterone between normal glands and pregnancy-dependent mammary tumors.*

INTRODUCTION

IN A PREVIOUS paper [1], progesterone was found to prevent the decrease in DNA synthesis of pregnancy-dependent mammary tumors after parturition in GR/A mice. On the other hand, estrogen had no such effect and the administration of estradiol benzoate in combination with progesterone did not stimulate the effect of progesterone on DNA synthesis. These findings indicate the importance of progesterone on the growth of this type of tumors during pregnancy. However, these results cannot exclude the possible participation of estrogen, since intact mice were used in the experiment [1] and therefore progesterone acted on the tumors in the presence of levels of endogenous estrogen in the circulation.

The present study was carried out to clarify more completely the relation between progesterone and estrogen on the growth of pregnancy-dependent mammary tumors in GR/A mice.

MATERIAL AND METHODS

Female mice of the highly inbred GR/A strain were mated with males at 60–70 days of age and were subjected to force-breeding without subsequent lactation. Only the animals with palpable mammary tumors during the 2nd and the 4th pregnancies were used. The day when the vaginal plug was found was designated as day 1 of pregnancy. Mice were divided into five groups; Group I received no treatment and served as the control. Groups II, III and IV were given daily subcutaneous injections of 2mg progesterone, 3mg progesterone and 3mg progesterone plus 0.5μg estradiol benzoate in combination, respectively, during days 14 and 19 of pregnancy and ovariectomized bilaterally on day 15. Group V received only unilateral

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ovariectomy on day 15. On the afternoon (about 3:00 p.m.) of day 19 of pregnancy, each mouse was given a single intraperitoneal injection of 50μCi [^{3}H] thymidine (5Ci/mmole; The Radiochemical Centre, Amersham, England) and was killed 2 hr later. [^{3}H]thymidine incorporated into DNA of normal and neoplastic mammary tissues was determined as described previously [2] as the index of DNA synthesis.

Number of mammary tumors and mammary tumor size expressed in terms of the geometric mean of the major two diameters were recorded on days 15 and 19 of pregnancy.

RESULTS

Changes in mammary tumor size during days 15 and 19 of pregnancy in each group is presented in Table 1. The average tumor sizes significantly increased in mice ovariectomized and injected with progesterone plus estrogen (Group IV) and mice ovariectomized unilaterally (Group V) ($P<0.01$). On the other hand, no further growth of tumors was observed in mice ovariectomized and injected with either 2 or 3mg progesterone singly (Groups II or III).

There was slight difference between groups in the number of fetuses at autopsy and the number of tumors changed little during days 15 and 19 of pregnancy in all groups.

Figure 1 shows [^{3}H]thymidine incorporated into DNA of normal and neoplastic mammary glands in each group. [^{3}H]thymidine incorporation was significantly lower in Groups II and III than in Group I not only in normal glands but also in tumors ($P<0.01$). Groups IV and V were at the levels similar to Group I and were significantly higher than Groups II and III in both measures ($P<0.01$). The incorporation of [^{3}H]thymidine into mammary tumors was significantly increased by the injection of 3mg progesterone as compared to the injection of 2mg progesterone, but this difference was not observed in normal glands.

The correlations in DNA synthesis between normal and neoplastic mammary glands were statistically highly significant in Groups I, IV and V ($P<0.01$).

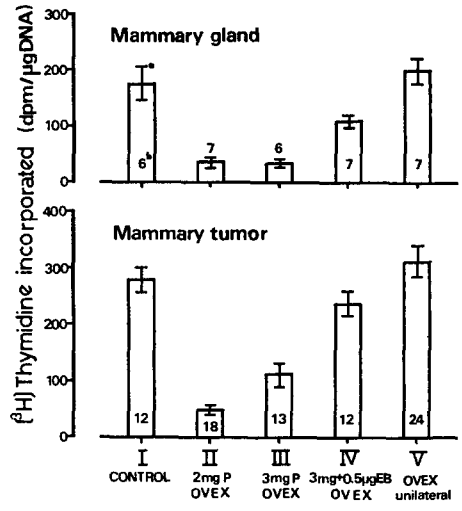

Fig. 1. *The* in vivo *incorporation of* [^{3}H] *thymidine into DNA of normal and neoplastic mammary glands on day* 19 *of pregnancy in each group. See Table* 1 *for details of treatments. Ovex: ovariectomy, P: progesterone, EB: estradiol benzoate. (a) Mean ± S.E.M. (b) Number of estimates. Significance of differences between groups are, Mammary gland:* I/II,III; II,III/IV,V; IV/V: $P<0.01$. *Mammary tumor:* I/II,III; II/III,IV,V; III/IV,V: $P<0.01$.

DISCUSSION

In the present study, DNA synthesis of normal and neoplastic mammary glands at the end of

Table 1. *Changes in the size of pregnancy-dependent mammary tumors in each group*

Group and treatment†	Number of mice examined	Number of fetuses	No. of tumors examined	Mammary tumor size (mm)‡ Day 15	Day 19	Changes (%)
I. Control	6	7·6 ± 1·0§	6	4·9 ± 0·6	5·1 ± 0·5	14·3 ± 4·5*
II. 2mg P, ovex	7	8·0 ± 1·0	14	4·1 ± 0·3	4·1 ± 0·3	5·7 ± 5·8
III. 3mg P, ovex	6	9·0 ± 0·7	14	3·7 ± 0·3	3·9 ± 0·4	4·1 ± 6·8
IV. 3mg P + 0·5µg EB, ovex	7	8·3 ± 0·9	15	3·8 ± 0·6	6·9 ± 0·8	87·1 ± 11·8***
V. Ovex unilateral	7	8·6 ± 0·6	12	3·8 ± 0·5	5·3 ± 0·4	78·6 ± 12·3***

†Each dose of progesterone (P) and estradiol benzoate (EB) were injected daily during days 14 and 19 of pregnancy. Ovariectomy (ovex) was performed on day 15. All mice were killed on day 19.

‡Mammary tumor size was expressed in terms of the geometric mean of the major two diameters. * and ***are different from zero at $P<0.05$ and 0.01, respectively.

§Means ± S.E.M.

pregnancy decreased significantly after bilateral ovariectomy despite the supplement with 2mg progesterone. Increase in the daily dose of progesterone to 3mg caused a significant increase in DNA synthesis in mammary tumors, but the values were still much lower than those of the controls. On the other hand, the administration of the same dose of progesterone (3mg) in combination with estrogen completely remedied the effect of ovariectomy on DNA synthesis, and raised levels to those of the controls. In accord with these results, progesterone combined with estrogen caused significant increases in mammary tumor size during the experiment as compared to the controls. In our previous study, estrogen was found to have no effect on DNA synthesis of mammary tumors in intact mice after parturition [1].

Sluyser and Van Nie [3] reported that the growth of transplanted mammary tumors elicited by progesterone and estrogen in castrated GR/A mice was stimulated by the treatment with progesterone and estrogen in combination, but it was much less sufficient by either hormone alone. These findings clearly indicate the permissive, but essential synergism of estrogen on progesterone in stimulating the growth of pregnancy-dependent or hormone-responsive mammary tumors in mice. There are indications that the synthesis of progesterone receptor in some tissues depends upon the presence of estrogen [4, 5]. Horwitz *et al.* [6] reported that 56% of human breast tumors which contained estrogen receptors also contained progesterone receptors, but progesterone receptors were absent in tumors which were devoid of estrogen receptors. Furthermore, Trams and Henning [7] found in carcinogen-induced rat mammary tumors that progesterone receptor concentration decreased very rapidly after ovariectomy. These data appear to suggest that in pregnancy dependent mammary tumors and in hormone-responsive mammary tumors estrogen induces the synthesis of progesterone receptor, thereby increasing the susceptibility of mammary tumor cells to progesterone.

Daily injections with 3mg progesterone to bilaterally ovariectomized mice enhanced DNA synthesis of mammary tumors, but not of normal glands. Addition of estrogen to progesterone increased significantly DNA synthesis in both normal and neoplastic glands. However, mice receiving progesterone plus estrogen had still significantly lower DNA synthesis in normal mammary glands than unilaterally ovariectomized mice, whereas there was no significant difference between these groups in the synthesis of mammary tumors. These observations have demonstrated the higher susceptibility to hormones in pregnancy-dependent mammary tumors than in normal glands.

Changes in mammary tumor size and changes in DNA synthesis in mammary tumors and normal glands were affected little by unilateral ovariectomy. This shows that the amount of ovarian hormones in the circulation is more than sufficient to stimulate the growth of normal or neoplastic glands.

Significant and positive correlations in DNA synthesis between normal and pregnancy-dependent mammary tumors in groups in which DNA synthesis of both glands was conspicuous support our previous hypothesis that this type of mammary tumors still retain more or less the intrinsic biological characteristics of normal mammary glands [1].

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Melphalan vs Polymelphalanum in Ovarian Cancer Patients Resistant to Cyclophosphamide: A Tentative Statistical Approach to Balance Risks and Benefits*

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Abstract—*A randomized trial based on a sequential analysis plan was started to check the activity of melphalan vs polymelphalanum in ovarian cancer patients resistant to cyclophosphamide. The trial was however closed in advance after treating only seven pairs of patients because of polymelphalanum toxicity. This decision is explained on statistical grounds in an attempt to provide a quantitative basis for balancing the toxicity found against the hoped-for benefits during the early phases of a clinical trial.*

INTRODUCTION

CYCLOPHOSPHAMIDE and melphalan are at present the two compounds which give the best response in ovarian cancer [1]. About 50% of patients treated with either melphalan [2] or cyclophosphamide [3] respond to the treatment. Very little information, however, is available on how to treat patients who are resistant to the first cycle of therapy.

Actinomycin D, 5FU and cyclophosphamide polychemotherapy showed some activity [2] but this was not confirmed by other authors [4]. High doses of cyclophosphamide showed greater toxicity and very little activity [4].

Among the many analogues of melphalan studied in the past [5, 6] a mixture of oligopeptides containing metaphenylalanine mustard (polymelphalanum) [7] has recently been marketed in Italy, with the trade name Peptichemio^{R} [8]. This compound has been extensively studied on cancer patients [9–11] and in a phase I trial on ovarian cancer it induced responses in 7 out of 15 patients who had never been treated before, and in 8 out of 26 patients treated before with different compounds [11]. These trials were not, however, carried out in controlled conditions.

Data on plasma cell myeloma showing that melphalan-resistant patients may respond to high-dose cyclophosphamide [12] suggested it was unlikely that cross-resistance would appear between the two drugs.

A trial was then started with the following aims: First, to explore whether cyclophosphamide-resistant patients obtained any improvement after melphalan or polymelphalanum treatment, and second, to check whether polymelphalanum had any advantage over melphalan treatment. A sequential trial was designed to compare the two drugs, should their efficacy be confirmed.

MATERIAL AND METHODS

Experimental plan

Patients entering the protocol had Müllerian ovarian cancer, histologically confirmed, stages III or IV of the FIGO classification, or abdominal and pelvic persistances. Maximum age was 75 yr. Cyclophosphamide, 100 mg/day, orally [3], was given for at least 2 months before patients were withdrawn as non-responders. Patients responding to cyclophosphamide were treated until relapse occurred. A few patients recorded as non-responders were in

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fact treated for more than 2 months, because subjective improvement was reported.

Patients were divided into three groups on histological grounds, i.e. serous, mucinous or undifferentiated carcinomas. Pairs were randomized in each of these groups to enter a sequential analysis plan based on 35% mean efficacy of the two treatments, and a minimal discriminating efficacy of 30%, to be obtained with a maximum of 33 pairs of patients. Response was graded by measuring two perpendicular diameters of at least one measurable mass, either by clinical examination, X-rays or ultrasound scanning. Complete response (CR) involved the disappearance of every measurable tumour mass for at least two months; partial response (PR) was achieved when a reduction of more than 50% was measured in at least one of the tumoral masses for at least two months; objective improvement (OI) when the reduction was from 25 to 50% for the same period of time. Non-responders (NR) were patients either with progressive or unchanged tumour [3].

Patients entering the second course of therapy at random, were treated as follows:

Melphalan group: 1 mg/kg, total oral dose, divided over 5 days, repeated once every 4 weeks [2].

Polymelphalanum group: 25 mg/m^2, i.v. on day 1; 50 mg/m^2 i.v. on days 2 and 3; 50 mg/m^2 i.v. every 2 weeks [11].

After two months of treatment patients were evaluated as described, and NR were withdrawn from the protocol.

Toxicity was scored as follows: (1) Allergic reaction; (2) Vomiting, (3) Alopecia; (4) Phlebothrombosis; (5) Mild leuk or thrombocytopenia; (6) leuk/or thrombocytopenia (WBC <2000, PL $<75{,}000$).

RESULTS

Table 1 reports the data collected on the seven pairs of patients studied before the protocol was closed for toxicity. One complete response was observed after melphalan treatment; this patient is still in remission after 16 months of treatment. No other response was observed during the two months of treatment, after which all the non-responders were withdrawn from the protocol.

The evaluation of one complete response out of 14 treated patients shows that the mean efficacy was already lower than the expected 35%; $P = 0{\cdot}05$, fiducial limits from 0·18 to 33·87%.

The sequential plan was therefore designed on an uncorrect hypothesis.

In addition, the two failure frequencies of 7/7 polymelphalanum and 6/7 melphalan tested by the one-way chi-square test against a theoretical frequency of 1/2, gave a highly significant ($P<0{\cdot}01$) probability of failure.

Evaluation of toxicity in terms of the number and severity of toxic events gave significant evidence of the higher toxicity of polymelphalanum in comparison with melphalan (Table 2) at the tested doses, which are those recommended by previous investigators. The protocol was consequently closed.

Table 1. *Patients submitted to the trial*

Pairs	Patient	Age	Staging	Cyclophosphamide		Second course	
				Total dose (g)	Response	Drug	Response
1	2525/73	53	S III	2	PR	MELPH	NR
	1411/74	64	S III	14	NR	POLIM	NR
2	3261/73	42	S III	37	PR	MELPH	NR
	2141/74	59	S III	143	OI	POLIM	NR
3	3639/74	59	S III	6	NR	MELPH	NR
	97/75	51	S III	5	NR	POLIM	NR
4	2451/74	75	S III	18·5	PR	MELPH	CR
	2506/74	59	S III	13	PR	POLIM	NR
5	3308/74	52	S III	15	PR	MELPH	NR
	3539/74	65	S III	10	NR	POLIM	NR
6	1512/75	41	S III	7	NR	MELPH	NR
	2446/74	47	S IV	28	PR	POLIM	NR
7	2718/73	51	U IV	15	OI	MELPH	NR
	2122/73	60	U III	23	PR	POLIM	NR

S = Carcinoma serous type.
I = Carcinoma undifferentiated type

Table 2. Toxicity by the treatments

Toxic events	Weight of toxicity	Melphalan No	Melphalan Total weight	Melphalan Risk (%)	Polymelphalanum No	Polymelphalanum Total weight	Polymelphalanum Risk (%)
Allergic reactions	1	0	0	<33*	1	1	0·36–58†
Vomiting	2	0	0	<33*	2	4	3·7–71†
Alopecia	3	0	0	<33*	2	6	3·7–71†
Phlebothrombosis	4	0	0	<33*	7	28	>67*
Leukopenia, mild	5	1	5	0·36–58†	0	0	<33*
Thrombocytopenia, mild	5	0	0	<33*	1	5	0·36–58†
Leukopenia <2,500	6	0	0	<33*	1	6	0·36–58†
Thrombocytopenia <75,000	6	1	6	0·36–58†	4	24	18–90†
Total		2	11		18	74	
% of toxic events		1–32†			68–99†		
Weight of toxicity (%)			7–22†			78–93†	

*P = 0·05 from Table 3.
†P = 0·05 confidence limits from binomial distribution tables.

The decision to close a protocol for toxicity is always somewhat subjective and evades any strict evaluation of the balance between observed toxicity and hoped-for benefits. From the statistical point of view, the fiducial limits of a percentage increase as the percentage approaches zero or 100%. For the zero frequency of responses, no fiducial limits can be calculated. It is, however, possible to evaluate the probability of not achieving any "response" phenomenon in a known number of samples, from a population containing a theoretical proportion of responses.

The probability of not observing any response (R) in a sequence of patients N with the risk of β = 0·05 is: $(1-P)^N = 0{\cdot}05$.

For a given value β this equation gives the value of N as a function of P (see Table 3 showing the hypothesis of efficacy E nullified at the stated probability in a sample N in which no response occurs): when no response is recorded in N samples, the efficacy is lower than the value given.

In the case of polymelphalanum treatment, at a 0·05 risk, the probability of achieving benefit was less than 33% after 7 non-responders, but already after 4 non-responders, the efficacy of the treatment was less than 50%.

The risk of toxicity, judging from the actual frequency of toxic events, was defined by the fiducial limits obtained from binomial tables. The figures at P = 0·05 were in a range from 0·36 to 58% for allergic reactions, low grade thrombocytopenia and serious leukopenia; from 3·7 to 71% for serious thrombocytopenia, from 47 to 100% for phlebothrombosis. In this case, however, the figure obtained from binominal distribution tables can give only a rough approximation since 7/7 (100%) cases are involved. It would, perhaps, be statistically more precise to ask what probability these patients have to escape phlebothrombosis. This probability, as set out in Table 3, is less than 33%.

Table 3. Number of patients (N) *not responding to treatment sufficient to nullify the hypothesis of efficacy* (E)

N	Efficacy % 0.05 = β	0.01 = β	0.15 = β
3	–	49	42
4	49	41	35
5	42	35	30
6	37	30	26
7	33	27	23
8	30	24	21
9	28	22	19
10	25	20	17
11	23	19	16
12	22	17	15
13	19	16	14
14	18	15	13
15	17	14	12
16	16	14	11
17	15	13	11
18	14	12	10

DISCUSSION

The present trial was closed because, at least for one of the drugs tested, toxicity appeared to be greater than expected benefits.

Analysis of the seven pairs of patients treated with melphalan or polymelphalanum led us to stop the trial when it was still too soon to draw any conclusions about the efficacy of melphalan.

The low toxicity of this treatment, and the one complete response obtained, suggested more patients might be admitted to the protocol to reach some conclusion in terms of efficacy.

The patients receiving polymelphalanum, however, were showing increasing cases of toxicity, while the hoped-for benefits did not appear.

Having calculated the probability of observing a benefit, which in fact was never observed in the present population, we could compare risks with benefits for polymelphalanum treated patients. The benefits expected, ie, objective improvement, partial or complete response lasting at least two months, were relatively small in relation to the toxicity risk, and an equally significant indication that treatment was not worth continuing could perhaps have been noted when with only four patients the probability of achieving benefits already fell below 50%.

In this trial the observation and treatment period was limited to two months, which was considered sufficient to detect treatment-induced responses and also to give some of the patients a chance for further treatment later.

Four patients withdrawn from the protocol but still eligible for chemotherapy were in fact treated with adriamycin, 40 mg/m^2, every three weeks. Three of these patients were from the polymelphalanum and one from the melphalan group. One partial response and one objective improvement were recorded.

In conclusion, the unsuccessful trial of polymelphalanum in ovarian cancer patients resistant to cyclophosphamide confirmed the utility of statistical probability tables to compare observed toxicity with expected benefits during the early phases of a trial, with a view to minimizing the risk for patients entering experimental protocols.

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The Effects of 1-β-D Ribofuranosyl-1, 2, 4-Triazole-3-Carboxamide (Ribavirin) on the Transplanted Tumours of Animals

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Abstract—*The effect of Ribavirin on the growth of three transplantable tumours was examined. At a concentration which was non-toxic to CBA mice, the compound reduced significantly the incidence of tumours in mice inoculated with transplanted adenovirus 12-induced tumours. Ribavirin was toxic for hamsters at a concentration of 100 mg/kg/day; at this concentration the compound reduced the tumour incidence of drug-treated animals inoculated with a transplanted SV40 virus-induced tumour, but the effect was less than that seen in mice. Studies in rats showed that Ribavirin had an inhibitory effect on tumour cell growth; however, drug-treated animals exhibited little or no lymphoreticular response to tumour growth, and this secondary immunosuppressive property of the compound had the collective effect of promoting tumour growth.*

INTRODUCTION

PREVIOUS studies have shown that several compounds, such as ethidium bromide, rifamycin and tilorone, inhibit the RNA dependent DNA polymerase essential for the replication of tumour viruses, and have an antitumour activity [1–4]; indeed, if the RNA viruses found in tumour cells are important in maintaining the cancerous properties of transformed cells, the two properties may be related. Thus, tilorone has a chemotherapeutic action against a wide range of viruses, and is an interferon inducer [5]; however, the antitumour activity of different analogues of this compound correlated more closely with the inhibitory activity on viral DNA polymerase, than with the ability to induce interferon [6]. From these observations, it is possible that some compounds active against RNA viruses may have antitumour activity.

The compound 1-β-D ribofuranosyl-1, 2, 4-triazole-3 carboxamide (Ribavirin) has been reported to inhibit the replication of both RNA and DNA viruses *in vitro* [7–9]. The compound acts by interfering with guanidine monophosphate formation and subsequent nucleic acid synthesis [10, 11]. In studies of influenza virus infection of chick cells, Ribavirin used at a concentration which completely inhibited influenza viral polypeptide production had no demonstrable effect on cellular protein synthesis [12]. In contrast, Ribavirin at a concentration which prevented influenza virus infection in ferrets suppressed the serum antibody response; the suppression of antibody formation was also demonstrated in guinea pigs immunized with influenza vaccine in Freund's adjuvant [13]. In the present studies, we report an investigation of the activity of Ribavirin against transplantable tumours, since the effect of this compound on either the RNA viruses of tumour cells or on the immune response of the host [14, 15] may initiate antitumour activity.

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MATERIAL AND METHODS

Ribavirin

Ribavirin was kindly supplied by Dr. R. W. Sidwell, ICN Nucleic Acid Research Institute, Irvine, California and by Lederle Labs., Gosport, Hants., and was stored at 4°C. The drug was weighed out freshly each day and dissolved in phosphate buffered saline, pH 7.2. For the treatment of animals, daily inoculations of 100 mg/kg were given subcutaneously to mice and hamsters, and daily inoculations of 12.5–100 mg/kg were given intraperitoneally to rats.

Tumour cells

Experiments were carried out in CBA mice, Syrian golden hamsters or in rats all of which

were obtained from closed, randomly-mated colonies at the University of Sheffield. The adenovirus 12-induced tumour of CBA mice [16], the SV40 virus induced tumour of hamsters [17], and the Rd3 tumour of rats [18] were maintained by transplanting tumour fragments at 2–3 week intervals into animals aged 4–5 weeks. To prepare tumour cell suspensions, subcutaneous tumours 10–15 mm in diameter were excised, freed from adhering fibrous tissue, minced to small fragments and treated with 0.25% trypsin to give a single cell suspension. The cells were washed with two changes of Eagle's minimal essential medium (MEM) containing 2% foetal calf serum and antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin) and the cell concentrations adjusted to a measured number of viable cells/ml; the cells were used immediately to inoculate animals.

Toxicity of Ribavirin for animals

For toxicity studies, Ribavirin was inoculated into groups of 9–10 mice or hamsters at a concentration of 50, 100 or 150 mg/kg; the compound was prepared fresh each day, and given by the subcutaneous route. Mice received a dose of compound daily for 20 days, and hamsters received a daily dose for 15 days. Due to the limited supply of the compound, similar studies could not be performed in rats. The number of dead animals was recorded daily, and changes in body weight were measured twice weekly; in addition, the surviving animals were all killed at the end of the treatment period and the spleen weights recorded.

Experimental design

(1) *Adenovirus 12-induced tumours.* Groups of CBA mice were inoculated subcutaneously with 100 mg/kg/day of Ribavirin. Two hours after the second drug inoculation, these animals, together with a group of untreated mice, were each inoculated subcutaneously with 5×10^5 transplanted adenovirus 12-induced CBA mouse tumour cells in 0.1 ml of MEM; the tumour cell inoculum used represented ten (50%) tumour-induced doses [16]. Ribavirin was given to the treated mice for a further 20 days, and the incidence and size of tumours in the animals was recorded twice weekly for four weeks.

(2) *SV40 virus-induced tumours.* Groups of hamsters of 90–110 g weight were inoculated subcutaneously with 100 mg/kg/day of Ribavirin. After the second injection of Ribavirin, these animals and a control group of untreated hamsters were each inoculated with $10^{2.0}$ viable SV40 virus-induced tumour cells per hamster; this tumour cell inoculum had been shown by prior titration to correspond to ten (50%) tumour-inducing doses. The drug-treated hamsters received 15 daily doses of Ribavirin and the incidence and size of tumours were recorded for 21 days at which time the experiments were terminated.

(3) *Rd3 tumours.* Groups of male rats of 250–300 g weight were each inoculated in the footpad with 5×10^6 Rd3 cells in an 0.1 ml volume. One group of rats received 100 mg/kg/day Ribavirin by the intraperitoneal route daily from two days before tumour cell inoculation; a second group received the same dose of Ribavirin from the same day that the tumour cells were given; and a third and fourth group received Ribavirin from two and four days, respectively, after tumour cell inoculation. The drug was given daily for eight days after tumour cell inoculation. A number of animals from each of the treated groups, and from an untreated control group, were killed at four days or eight days after inoculation with tumour cells. From these rats, the footpad and popliteal lymph nodes were taken for histological examination, as described previously [18]. In a second experiment, groups of rats were inoculated daily by the intraperitoneal route with Ribavirin at different concentrations from two days before footpad inoculation with 5.0×10^6 Rd3 cells. Animals from each group were killed at 4 or 8 days after tumour cell inoculation and specimens taken for histological examination, as described above.

The size of the lymph nodes was measured at autopsy, and the presence and relative number of tumour cells in the lymph nodes was determined by microscopic examination; these latter results were graded according to the following scheme:

Grade 0	—	no tumour cells present.
Grade 1	—	tumour cells present in the subcapsular sinus only.
Grade 2	—	tumour cells in subcapsular sinus and radial sinusoids.
Grade 3	—	massive replacement of node with tumour cells.

In addition to the above observations, the size of the footpad tumours was measured using calipers, and these primary tumours were examined histologically. The presence of macrophages and lymphocytes in the tumours or metastases was recorded, as an indication of a cellular immune response; in control animals,

the presence of a lympho-reticular response was a marked feature of the primary tumour.

RESULTS

Toxicity studies

The toxic effect of Ribavirin was investigated in both CBA mice and hamsters. The results are shown in Table 1. At concentrations of 50, 100 and 150 mg/kg/day, Ribavirin did not cause death in any of the mice tested; however, 100 mg/kg/day and 3 out of 10 hamsters given 150 mg/kg/day died during the study period. The body and spleen weights of these two groups of treated hamsters fell significantly as a result of Ribavirin treatment (Table 1).

Effect of Ribavirin on an adenovirus 12-induced tumour of CBA mice

Two experiments were carried out which showed very similar findings; the results are given in Table 2. In both experiments, palpable

Table 1. Toxic effect of Ribavirin on CBA mice and on hamsters

	CBA mice (20 doses)			Hamsters (15 doses)		
Drug concentration (mg/kg/day)	No. of dead animals*	Body weight change (mean) (%)	Spleen weight change (mean) (%)	No. of dead animals	Body weight change (mean) (%)	Spleen weight change (mean) (%)
50	0/9	+56†	+59	0/10	+11	+ 5
100	0/9	+17	+44	1/10	− 4	− 8
150	0/9	+ 6	+12	3/10	−26	−18
Nil	0/9	+34	+18	0/10	+22	+ 6

*No. of dead animals/total of animals tested.
†Positive sign denotes weight gain; negative sign denotes weight loss.

mice given 100 or 150 mg/kg/day failed to gain weight to the same extent as control animals, though the compound did not significantly affect spleen weight. None of the hamsters inoculated with Ribavirin at 50 mg/kg/day died during the three week treatment and observation period. The body weight of these animals increased by 11% of the initial weight and the spleen weights increased by 5%; these values compare with 22 and 6% respectively, for control hamsters. One out of ten hamsters given

tumours were first observed in untreated mice 14 days after inoculation with 5×10^5 viable tumour cells. At 26 days after tumour cell inoculation, a total of 15 of 16 (94%) mice in the two experiments had rapidly enlarging tumours; the animals were killed at this time, since previous experience had shown that these animals would die with massive growth within the following 5–7 days. In contrast, mice treated with 100 mg/kg/day of Ribavirin from one day before tumour cell inoculation developed

Table 2. Effect of Ribavirin on transplanted adenovirus 12-induced tumours of CBA mice

Exp. No.	Ribavirin treatment	Incidence of tumours—days, post-inoculation (mean diameter of tumours in cm)					
		7	14	17	21	24	26
1	100 mg/kg daily from day 1 to day 21	0/8	0/8	1/8 (0.2)*	2/8 (0.2)	4/8 (0.4)	4/8 (0.7)
	Nil	0/8	2/8 (0.3)	4/8 (0.3)	5/8 (0.7)	6/8 (1.0)	7/8 (1.5)
2	100 mg/kg daily from day 1 to day 21	0/8	0/8	0/8	2/8 (0.1)	5/8 (0.3)	5/8 (0.5)
	Nil	0/8	2/8 (0.1)	4/8 (0.3)	5/8 (0.5)	8/8 (1.0)	8/8 (1.4)

*No. mice with tumours/No. mice inoculated (mean tumour diameter in cm).

significantly fewer tumours than observed in control mice. Thus, at 14, 17 and 21 days, post-inoculation, tumours were palpable in 0, 1 and 4 Ribavirin-treated mice, respectively, whilst in control mice the incidence of tumours was 4, 8 and 10 at the above times. The results at 26 days, post-inoculation, showed that Ribavirin-treated mice had significantly less tumours than control animals ($X^2 = 4.218$, $P = <0.05$). In addition, at the end of the experiments, the tumours present in treated mice had a mean diameter of 0.5–0.7 cm, whilst at this time the tumours of control animals had a mean size of 1.4–1.5 cm. Thus, Ribavirin treatment inhibited tumour formation, and the growth of those tumours which did appear (Table 2).

Effect of Ribavirin on SV40 virus-induced tumours of hamsters

The effect of Ribavirin on the transplantability and growth of SV40 virus-induced tumours of hamsters is shown in Table 3. For control animals, palpable tumours were first observed at 12 days post-inoculation; all hamsters had recognisable tumours at 16 days which grew rapidly. In contrast, four of ten hamsters treated with 100 mg/kg/day had palpable tumours at 12 days post-inoculation, and only seven developed tumours during the observation period. In addition, the mean size of tumours at 20 and 23 days was less for treated hamsters than for control animals. The small number of animals used in this experiment precluded tests of significance on these results.

Table 3. Effect of Ribavirin on transplanted SV40 virus-induced tumours of hamsters

Ribavirin treatment	Incidence of tumours—days, post-inoculation (mean diameter of tumours in cm)				
	9	12	16	20	23
100 mg/kg daily from day 1 to day 23	0/10	4/10 (0.3)*	7/10 (0.6)	7/10 (1.8)	7/10 (2.7)
Nil	0/10	9/10 (0.2)	10/10 (0.7)	10/10 (2.3)	10/10 (3.7)

*No. of hamsters with tumours/No. of hamsters inoculated (mean tumour diameter in cm).

Effect of Ribavirin on Rd3 tumour cells of rats

(a) *Effect of dose of Ribavirin.* At four or eight days after Rd3 tumour cell inoculation and treatment with varying doses of Ribavirin, groups of rats were killed, and the local tumours and regional popliteal lymph nodes excised; sections from these tumours were stained by H and E for microscopic examination. The results are shown in Table 4. At four days after tumour cell inoculation, tumours were well established in the footpads of control rats and in animals treated with 25 mg/kg of Ribavirin, but only a few tumour cells were present in the footpads of rats treated with 100 mg/kg of the compound. Whereas the tumours of control rats showed a marked lympho-reticular response with extensive lymphocyte and macrophage infiltration in and around the tumours, the tumours of treated animals showed no evidence of a cellular immune response (Fig. 1). Metastasis of the Rd3 tumour cells to the popliteal lymph node was found to have occurred in both treated and untreated rats; evidence of a cellular immune response was seen in the nodes of control rats, but none was found in those of treated rats (Fig. 1).

At eight days after inoculation, the footpad tumours of control rats were very large, and histologically showed massive tumour cell growth with lymphocyte and macrophage infiltration (Table 4). The tumours of rats treated with 25 mg/kg/day of Ribavirin were similar in size but those of rats treated with 100 mg/kg/day of the drug were smaller; the tumours of all treated rats showed little or no histological evidence of a lympho-reticular response. Similar findings were seen in the popliteal lymph nodes of these animals. Thus, the tumour metastasised to the lymph nodes of rats treated with 100 mg/kg/day of Ribavirin and to the nodes of control animals; however, the number of tumour cells was less in treated than in control rats, and there was no demonstrable cellular immune response (Table 4).

(b) *Effect of time of treatment.* Since any alteration in the pattern of Rd3 tumour development in Ribavirin-treated rats was seen best in

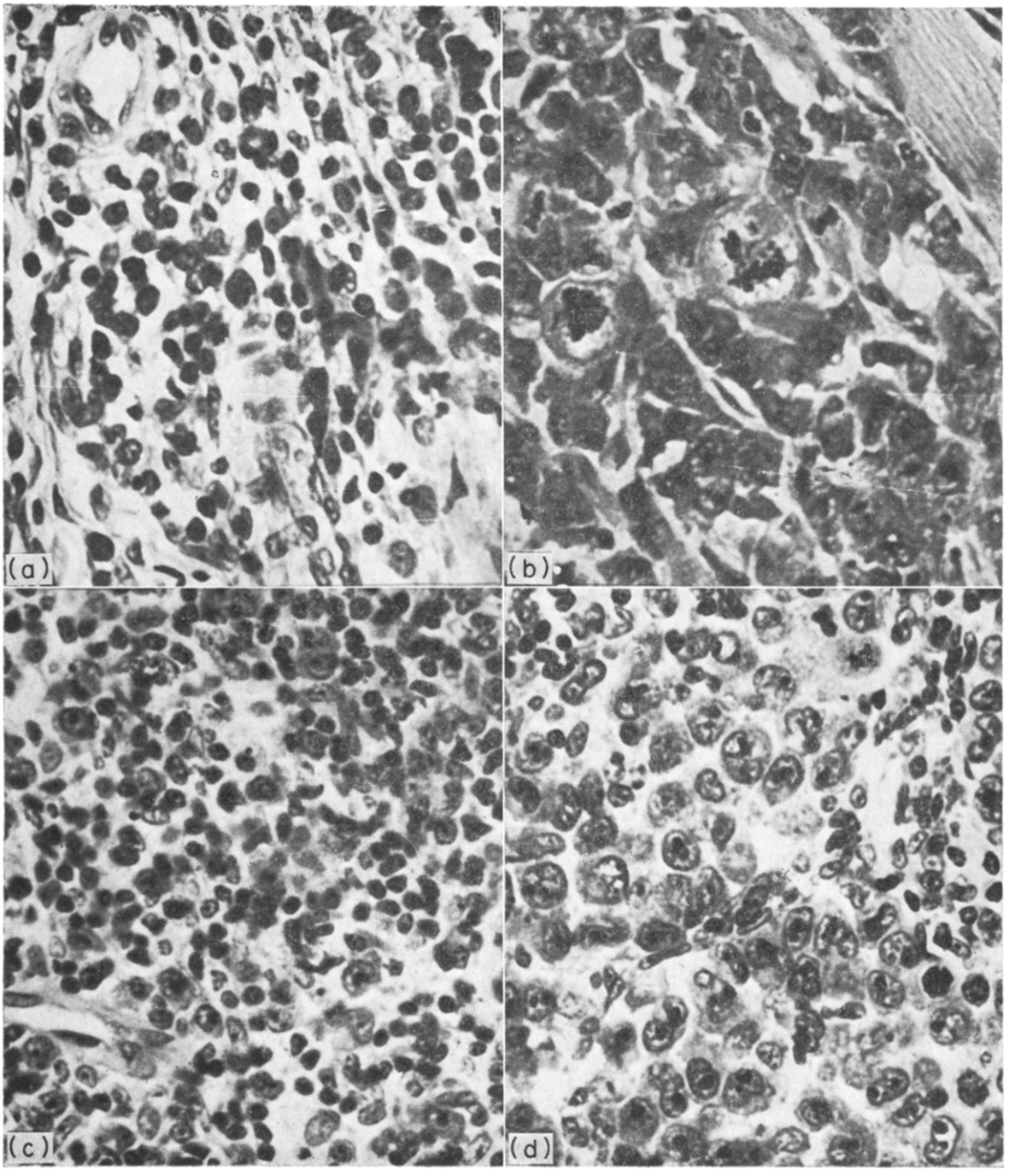

Fig. 1. *Histological appearance of Rd*3 *tumours in rats.* (*a*) *Tumour and lymphoid cell in primary tumour.* (*b*) *Tumour cells only in primary tumour of rat treated with* 100 mg/kg *Ribavrin.* (*c*) *Tumour metastasis in a regional popliteal node with tumour and lympho-reticular cells.* (*d*) *Tumour metastasis in popliteal node with tumour cells only.* (*Magnification* × 700).

Table 4. Effect of Ribavirin on the growth and metastasis of Rd3 tumour cells in rats

Group	Ribavirin treatment (mg/kg/day)	Response of rats to inoculation with $5 \times 10^{6.0}$ Rd3 tumour cells							
		4 days, post-inoculation				8 days, post-inoculation			
		Footpad		Popliteal lymph node		Footpad		Popliteal lymph node	
		tumour*	LRR†	tumour‡	LRR	tumour	LRR	tumour §	LRR
1	100	+	–	1.25 (0.2)	–	++	–	9.8 (1.25)	–
2	50	NT	NT	2.0	NT	NT	NT	(1.80)	NT
3	25	++	–	2.0	–	+++	±	20.0 (1.90)	±
4	12.5	NT	NT	2.0	NT	NT	NT	NT (2.4)	NT
5	Nil	++	++	2.0	+	+++	++	17.5 (2.5)	++

*Tumour growth in footpad; +, few tumour cells present; ++, tumour well established; +++, gross tumour growth.
†LRR—lympho-reticular response; –, no LRR in or around tumour; +, occasional lymphocyte and/or macrophage; ++, marked LRR.
‡Mean grade of metastases (range of response).
§Mean diameter of node (mean grade of response) in mm^2.

animals treated with 100 mg/kg/day, this dose was given to five groups of rats. Tumour cells were inoculated into the footpad two days after treatment started (group 1), on the day treatment started (group 2) or two or four days before treatment with Ribavirin (groups 3 and 4). The results are shown in Table 5. At 4 days after tumour cell inoculation, the findings in control and drug-treated rats were very similar. Thus, tumours were established in the footpads, though less well in animals treated from two days before tumour cell inoculation, and metastases were seen to the lymph nodes. Animals treated with Ribavirin showed no evidence of a

Table 5. Effect of Ribavirin given at different times on growth of Rd3 tumours in rats

Group	Ribavirin treatment (100 mg/kg/day from day –)	Response to Rd3 tumour cell inoculation								
		4 days, post-inoculation				8 days, post-inoculation				
		Footpad		Popliteal lymph node		Footpad		Popliteal lymph node		
		tumour*	LRR†	tumour‡	LRR	tumour*	LRR	tumour	size of node mean (mm^2)	LRR
1	–2	+	–	1.5 (1–2)	–	++	–	1.0 (0–2)	7.7	–
2	0	NT	NT	1.5 (0–2)	–	NT	–	1.25 (0–2)	10.5	–
3	2	++	–	2.0 (1–3)	–	++	±	2.0	18.2	±
4	4	NT	NT	NT	NT	NT	±	1.3 (0–2)	26.3	+
5	Nil	++	+	1.5 (0–2)	+	±	+–+	Nil	23.5	+++

*Tumour growth in footpad; +, few tumour cells present; ++, tumour well established; +++, gross tumour growth.
†LRR—lympho-reticular response; –, no LLR in or around tumour; +, occasional lymphocytes and/or macrophage; ++, marked LRR.
‡Mean grade of metastasis (range of response).

lympho-reticular response in the popliteal lymph node, but the metastases of control rats showed macrophage infiltration in and about the tumours (Table 5).

At eight days after Rd3 tumour cell inoculation, the findings for control and drug-treated rats were quite distinct (Table 5). The footpads of control rats contained only a small number of live tumour cells, but the specimens showed an extensive lympho-reticular response; no tumour cells were found in the popliteal lymph nodes of these animals but again a marked macrophage infiltration was observed. Compared to the findings at four days, post-inoculation, these results clearly indicate that the tumour cells were being rejected. In contrast, the primary tumours of drug-treated rats were well established and showed little or no cellular immune response; by comparison with the results seen in control rats, the effect of Ribavirin treatment had been to inhibit the immune response and promote tumour development. The size of the popliteal lymph nodes at eight days after tumour cell inoculation differed markedly for the different groups. Thus, the nodes of control rats were very large but contained no recognisable tumour cells; histologically, the increase in size was due entirely to the lympho-reticular response. The popliteal nodes of treated rats all showed evidence of tumour cell metastasis. The nodes of rats treated with Ribavirin for the longest period of time showed no cellular immune response, whilst the nodes of other treated groups showed a minimal response. Thus, the node sizes were inversely related to the length of Ribavirin treatment (Table 5).

DISCUSSION

The demonstration that some antiviral compounds which are active against viruses present in tumour cells may also have antitumour activity [1, 2] has two important implications. Firstly, the finding suggests that the activities of tumour viruses are important in the maintenance of the neoplastic properties of tumour cells; compounds which interfered with the function of these oncornaviruses may act also on the tumour cells that contain them. Secondly, the possibility exists that in the lists of compounds which have known antiviral activity, there may be found drugs which have activity against tumours. For this reason, we have investigated the antitumour properties of Ribavirin. This compound has been shown to inhibit both RNA and DNA viruses *in vitro* [7, 9], and *in vivo* [8, 13].

The transplanted adenovirus 12-induced tumour of CBA mice is a rapidly growing, undifferentiated tumour [19]: the tumour is encapsulated, and shows no evidence of metastasis. The present results show that Ribavirin, at a concentration of 100 mg/kg/day inhibited the growth of this tumour; both the number of tumours which developed and the rate of tumour growth were significantly reduced by Ribavirin in two separate experiments. Although it has been shown that Ribavirin has an immuno-suppressive effect on the host at the concentration used. [13], and that mice bearing adenovirus 12-induced tumours exhibit a marked cell-mediated immune response [16, 20], the sum total effect of the compound was to limit but not totally inhibit tumour growth. This may be due in part to the tolerance of mice for Ribavirin, and the relatively minor effect of the compound on the immune response of this species (13); thus, at 100 mg/kg/day mice were not killed, and although body weight was reduced the spleen weights increased.

The results obtained for the effects of Ribavirin on a transplantable SV40-induced tumour of hamster was similar to those for the adenovirus 12-induced tumour. This tumour is a very rapidly growing anaplastic carcinoma, the cells of which did not metastasise when inoculated subcutaneously; however, metastasis to regional lymph nodes could be demonstrated following footpad inoculation [21]. Ribavirin at a concentration of 100 mg/kg had a marginal effect on the incidence and growth of transplanted SV40 virus-induced tumours of hamsters; at this concentration the compound was clearly toxic to hamsters causing death in some animals, and a reduction in both body and spleen weight. Since this tumour has been shown to be immunogenic [22], the relative failure of Ribavirin compared to the results obtained for adenovirus-induced tumours may be due to the greater effect of the compound on the immune mechanism of the host.

The Rd3 tumour of rats is a chemically-induced tumour which metastasises to the lymph nodes following footpad inoculation [18, 21]. Using different doses of Ribavirin to treat rats inoculated with Rd3 tumour cells, the results showed that all rats developed footpad tumours, but the size of the tumours and the lymph node metastasis were measurably reduced in rats given the compound at a concentration of 100 mg/kg/day; the reduction in size was probably due to the absence of a lympho-reticular response in the tumour mass. In a further experiment, the effect of Ribavirin

on the immune response is more clearly demonstrated; thus, the tumour cells inoculated into control rats were almost entirely rejected at eight days, post-inoculation. The number of cells necessary to produce a tumour with Rd3 cells is critical, and in this experiment the number used was probably too small. In contrast, treated animals given the same tumour cell dose had established tumours at eight days after inoculation; there was little or no demonstrable cellular immune response in those hamsters or in the metastasis which was probably the reason why the tumours were not rejected in the same manner as seen in untreated rats.

The present results indicate that Ribavirin was active against the cells of three transplantable tumours; however, the concentration of compound used to demonstrate this activity was also immunosuppressive. This latter activity was probably least important in the experiments in mice using the adenovirus 12-induced tumour, since this host was relatively tolerant to Ribavirin. In contrast, the antitumour effects of Ribavirin was less evident for the other two tumour systems investigated, where the effect of the compoud on the immune response of the host abrogated the effect of the compound on the tumour cells. The above experiments indicate that Ribavirin probably has no value in antitumour therapy, and may even have deleterious effects; however, antiviral compounds which do not affect the immune response may yet be of value, and these should be investigated.

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A Fine Structural Study on the Therapeutic Effect on an Aromatic Retinoid on Chemically-Induced Skin Papillomas of the Mouse

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Abstract—*Skin papillomas induced by dimethylbenzanthracene and croton oil were treated systemically with a single injection of an aromatic new retinoid (Ro 10-9359). A marked regression of the tumors occurred within 7 days. The effects of this drug as seen with the electron microscope were (a) a marked enhancement of production of mucopolysaccharides and their secretion into the extracellular space, (b) a labilization of the plasma membrane resulting in vacuolization and loss of cytoplasmic constituents into the extracellular space, (c) an increase in lysosomal organelles, (d) formation of many necrotic cells. There were no clear-cut effects on either the nuclei, nucleoli or cytoplasmic constituents such as ribosomes and tonofibrils, nor was there any increase in the number of gap junctions as seen in keratoacanthomas after local retinoic acid treatment. Our interpretation favours the hypothesis that cell necrosis is mainly due to the labilization of the plasma membrane, but that other effects like the enhanced mucopolysaccharide production secreted into the extracellular space, might also play a role in the regression of the papillomas.*

INTRODUCTION

VITAMIN A is important for physiological functions such as growth, vision and fertility and for the differentiation of epithelial tissues. Possibly linked to this latter function vitamin A and its analogs (retinoids) also show the capacity to prevent and revert hyper- and metaplastic- changes *in vitro* [1–3] and even neoplastic lesions *in vivo* [4–13] induced either by vitamin A deficiency or by carcinogens. The mechanism of action is not known but several phenomena should be considered such as the labilization of lysosomal membranes by vitamin A [14], the fragmentation of the plasma membrane [15], the generation of gap junctions [16].

The model used in this study was the skin papilloma of the mouse induced by carcinogens [7]. It is a benign tumor with a potential malignant degeneration. It is characterized by hyperplastic and metaplastic changes. These papillomas decrease rapidly in size when treated systemically with a single injection of a new aromatic retinoic acid analog (Ro 10-9359).

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A previous study [17] had already shown that the mechanism of action for this analog was not to inhibit DNA synthesis but to induce necrosis. In the present study we used electron microscopy and histochemical staining procedures to define the cytological parameters accompanying the regression of these papillomas in order to gain more insight into the mechanism of action of these interesting compounds.

MATERIAL AND METHODS

1. *Animals*

Female Swiss albino mice of the Füllinsdorf random-bred strain were used. They were fed on the mouse diet Nafag 199, containing 2500 I.U. of vitamin A per kg of food.

2. *Tumors*

Skin papillomas were induced by skin painting with 7,12-dimethyl benzanthracene (DMBA) and croton oil according to the method described elsewhere [5, 7]. Twenty mice with 2–3 papillomas measuring between

3 and 8 mm in diameter were selected for the experiment.

3. *Treatment*

The retinoid, ethyl all-trans-9-(4-methoxy-2, 3, 6-trimethylphenyl) 3, 7-dimethyl-2, 4, 6, 8-nonatetraenoate (Ro 10-9359) was suspended in arachis oil (250 mg/ml) and given intraperitoneally to the animals in a single dose of 1000 mg/kg. In previous experiments this substance was shown to be highly effective in causing regression of papillomas [7]. In the present experiments the regression was determined by measuring the percentage decrease of the volume of the papillomas [6] from the time of injection until the time of sacrifice. Time intervals studied between treatment and sacrifice were 8 hr, 16 hr, 2 days, 3 days and 7 days. The total volume changes of the tumors were measured and expressed as percentage change of the volumes of the tumors per animal [6].

4. *Electron microscopy*

Animals were killed by cervical dislocation and small pieces of skin and papillomas were sampled and immersed for 24 hr in the following fixative: 1% glutaraldehyde (Serva, Heidelberg, Western Germany), 4% paraformaldehyde (Merck, Darmstadt, Western Germany) buffered with 0·1M phosphate buffer, pH 7·2. The blocks were rinsed overnight with phosphate buffer and postfixed for 2 hr with 2% osmium tetroxide in 0·1M cacodylate buffer, pH 7·2. Dehydration in a graded alcohol series was followed by epon embedding [18]. The sections were cut with diamonds on a Porter-Blum ultra-microtome MT-1 (Ivan Sorvall, Inc., Norwalk, Connecticut, USA) and stained with an aqueous 5% uranylacetate solution followed by lead citrate [19]. They were examined in a Philips EM 300 electron microscope (Philips, Eindhoven, The Netherlands).

5. *Histochemistry*

Acid mucopolysaccharides were stained on formalin fixed sections with the dialysed iron method according to Hale [20].

RESULTS

1. *The untreated papilloma*

Light microscopy. These tumors are mainly characterized by a thickening of all cell layers of the epidermis. The number of nuclear layers is increased from 1–3 in the normal skin to 7–12 in the papilloma. The histological features of normal skin are, however, fully preserved. The keratinization varies only slightly: The keratinized layer in most places is very thick but the cohesion of the cells appears to be lower. The dead cells defoliate easily retaining a spiny appearance. There are usually no signs of inflammation or necrosis, only the network of the many blood vessels and lymphatics in the subcutis beneath the papilloma is striking. Probably due to lack of space during the development the tumor acquires a deeply infolded, sometimes even convoluted aspect with keratin encapsulated by the epithelium.

Electron microscopy. Papillomas retain also at the ultrastructural level all features of normal skin. In addition the usual signs of a hyperplastic state are present: Big nuclei with euchromatin and huge nucleoli, large masses of polyribosomes in the cytoplasm with a usually well-developed Golgi apparatus.

2. *Papillomas treated with Ro* 10-9359, 8 *and* 16 *hr after injection*

Light microscopy. At these time intervals there are no appreciable changes with respect to the untreated papilloma. There is also practically no volume change of the tumors.

Electron microscopy. No conspicuous alterations were detected at either time interval.

3. *Papillomas treated with Ro* 10-9359, 2 *and* 3 *days after injection*

Light microscopy. At this stage tumors decreased in volume considerably in our experiments by 33, 33 and 46% at 48 hr, by 42, 45, 48, 48 and 80% at 72 hr. The effects of the treatment are distributed unevenly. Some regions of the papilloma are unaltered whereas others show distinct degenerative signs: large inclusion bodies, vacuolization, pyknosis and karyorrhexis.

Other regions form a homogeneous necrotic mass where no cytological features can be distinguished any more.

Electron microscopy. Necrotic cells are sharply distinguishable from the relatively intact cells. They are easily recognizable by their 'empty' cytoplasm (Fig. 6), the occasional large secondary lysosomes (Figs. 5 and 6), the nearly absent ribosomes, and in more advanced cases, by the fragmented or pyknotic nucleus. The rest of the cells shows more subtle alterations: there is a conspicuous widening of the intercellular cleft (Figs. 1–5), which is due to the presence of (a) a finely fibrillar mass, (b) many smooth vesicles and vacuoles (Figs. 2–5), (c) other cytoplasmic constituents such as ribosomes, tonofilaments,

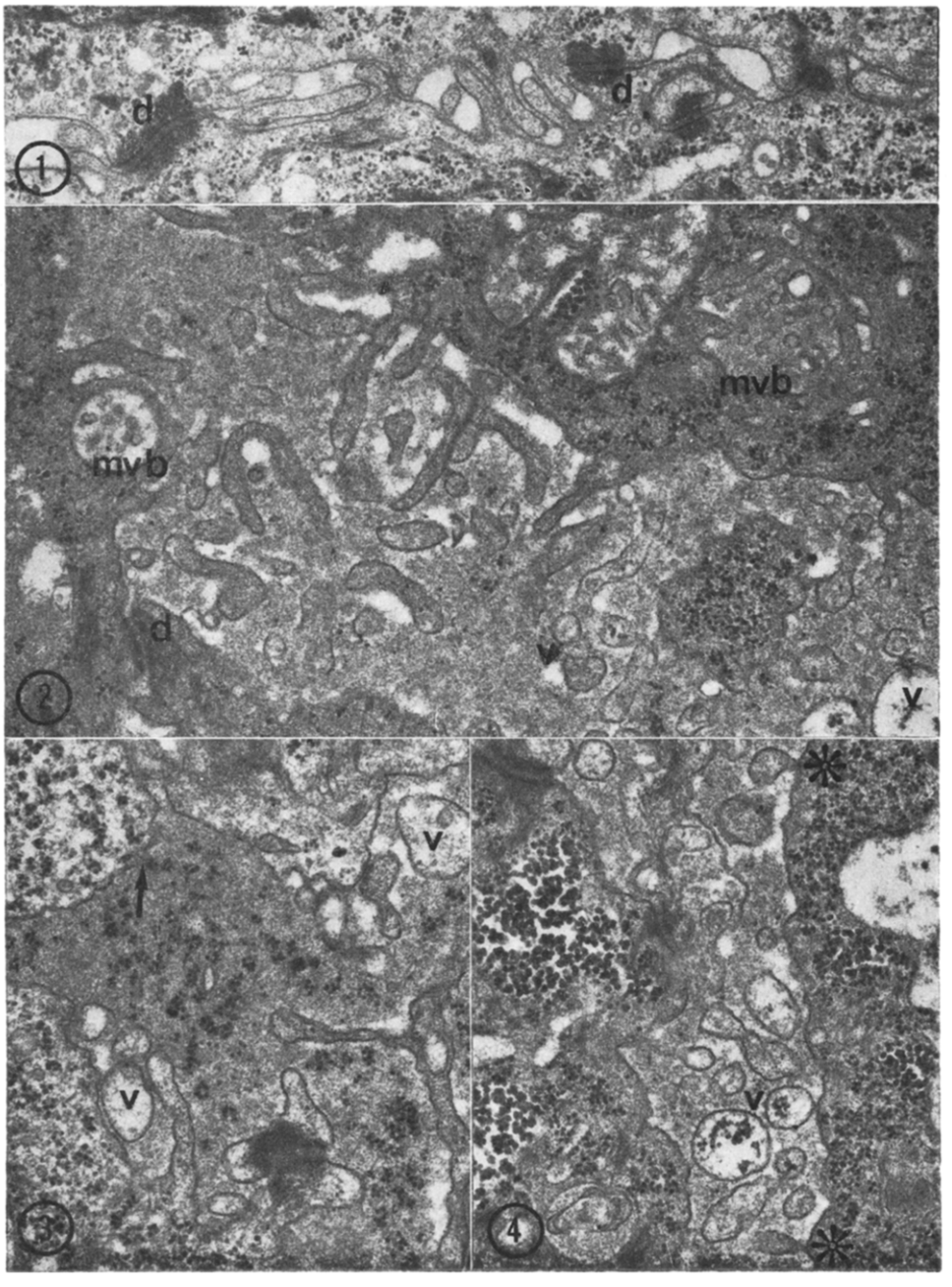

Fig. 1. *Intercellular cleft of the basal layer of a papilloma* 48 hr *after treatment. This part appears very similar to the untreated papilloma at the* 8 *and* 16 hr *interval after treatment. There is a slight widening of the cleft and at a few places there are small deposits of finely fibrillar materials. d desmosomes.* × 28,800.

Fig. 2. *Intercellular space of the basal layer of a papilloma* 48 hr *after treatment. In this case the intercellular cleft is widened enormously and filled with a dense, finely fibrillar material. The plasma membrane protrudes and forms elongated processes. Some vesicles appear to be derived from these processes. Note also* 2 *multivesicular bodies (mvb). d desmosomes.* × 30,600.

Fig. 3. *Same material as in Fig.* 2. *This figure shows that the damage to the plasma membrane can lead to the outflow (arrow) of (poly) ribosomes. Again, the formation of vacuoles (v) can be seen. There is also finely fibrillar material present which by histochemical staining methods (dialysed iron method of Hale) contains acid mucopolysaccharides (see Fig.* 8). × 29,250.

Fig. 4. *Same material as in Fig.* 2. *This figure shows that the vacuoles (v) are derived from protrusions of the intercellular cleft by a pinching off (*). Ribosomes and other cytoplasmic material is often trapped in these vacuoles.* × 30,600.

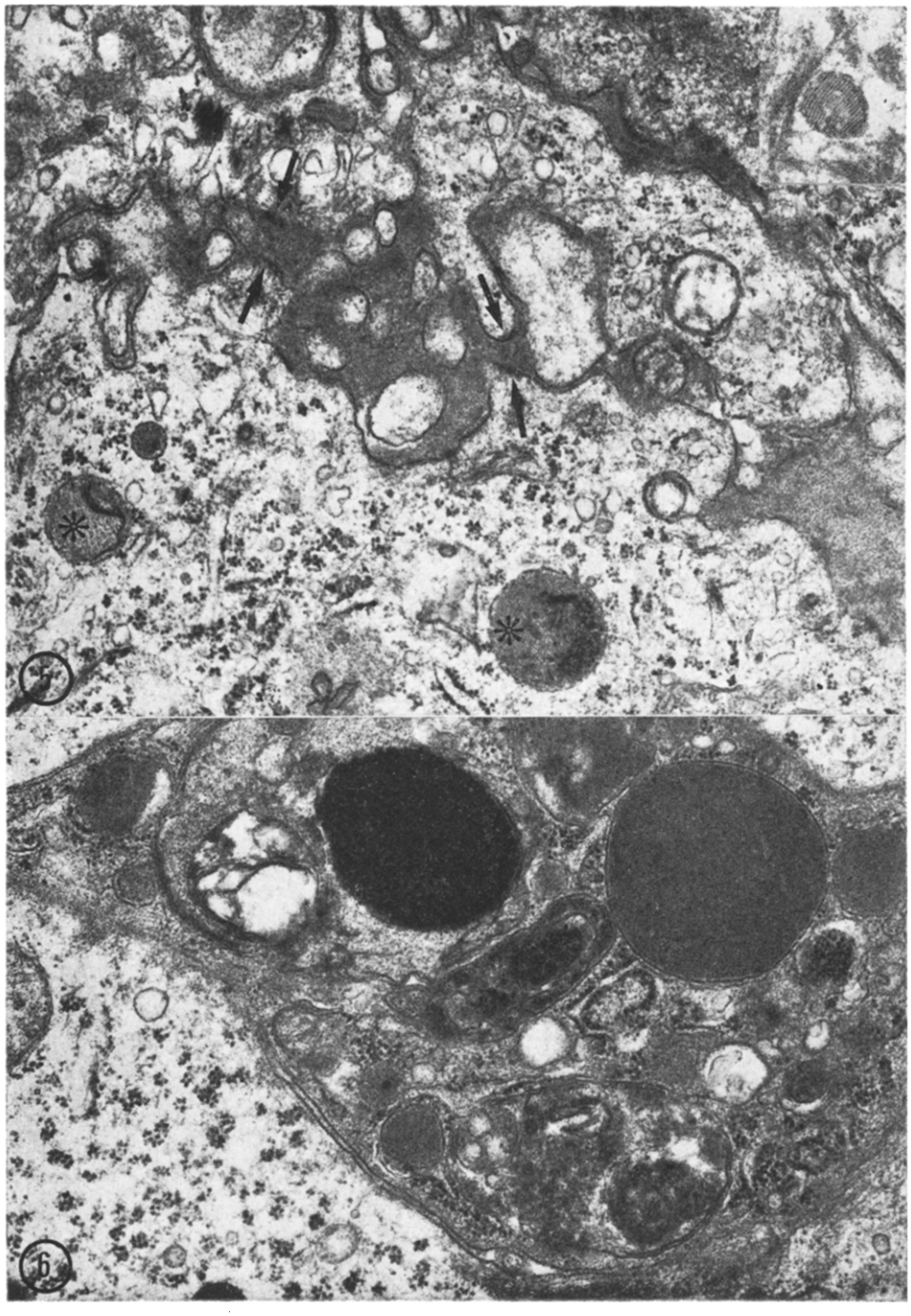

Fig. 5. *Basal layer of a papilloma* 72 hr *after treatment. At this stage the formation of vacuoles in the intercellular space has further progressed. The intercellular material acquires a denser appearance. Desmosomes are broken (arrows). Note also two secondary lysosomes* (*). × 28,980. *inset: inclusion body with membraneous material.* × 61,200.

Fig. 6. *Same material as in Fig.* 5. *Degenerating cell containing huge secondary lysosomes. On either side of this cell degenerating cells are found with a swollen, 'empty' cytoplasm.* × 30,150.

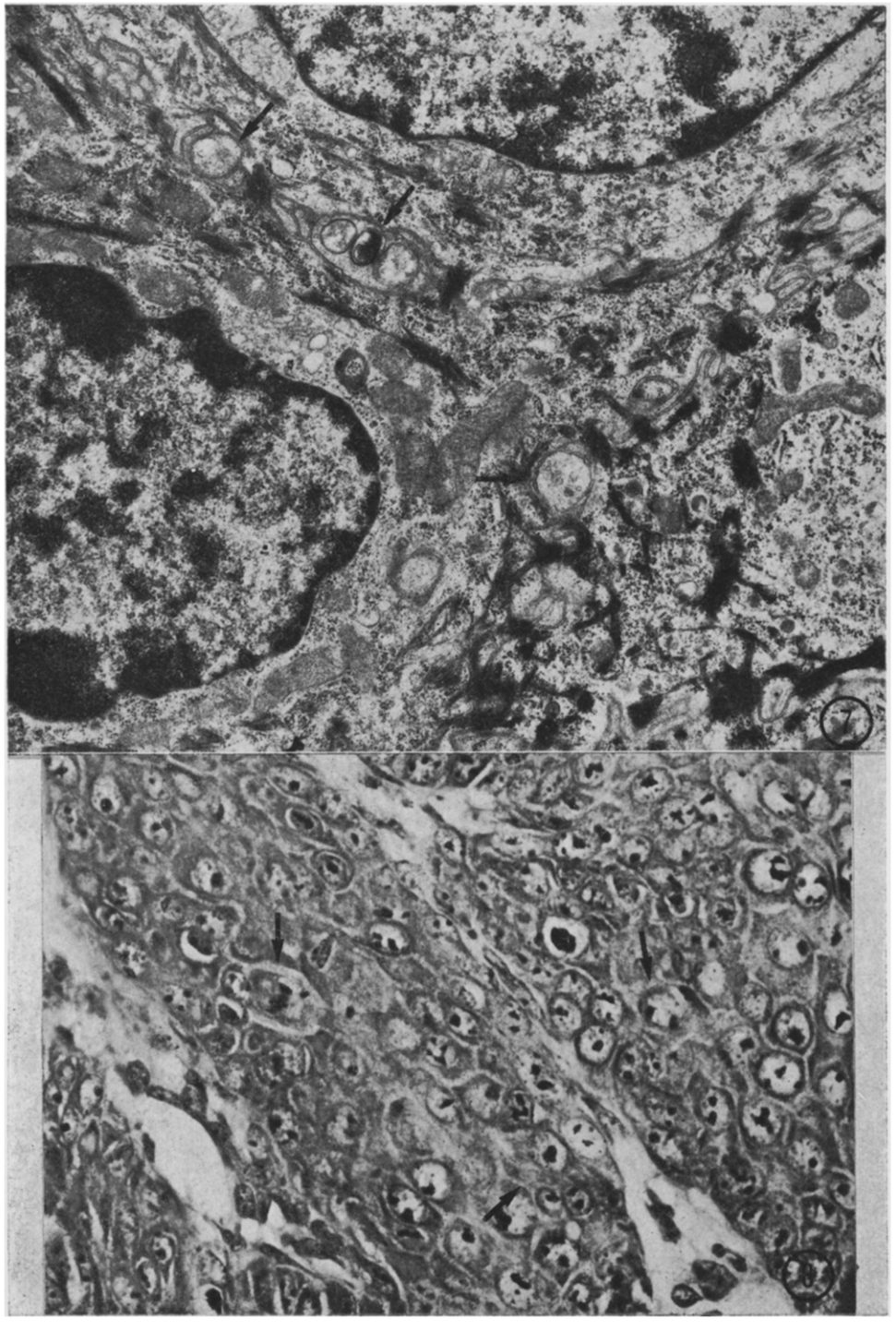

Fig. 7. Skin, basal layer, 72 hr *after retinoid treatment. The skin preserves its integrity throughout the treatment with Ro* 10-9359, *except that in few instances there are vacuoles containing cytoplasmic constituents in the intercellular cleft (arrows).* × 16,650.

Fig. 8. Basal layers of a skin papilloma, 72 hr *after treatment. Histochemical demonstration of acid mucopolysaccharides in the intercellular space (arrows) by means of the method of Hale* (20). × 576.

glycogen (Fig. 3), etc. The vacuoles seem to be formed by a pinching-off from protrusions of the plasma membrane (Figs. 2–4). Smooth and rough vesicles probably derived from the Golgi apparatus communicating with the extracellular space are involved in the secretion and possibly reabsorption of the extracellular mass. This latter mass consists of mucopolysaccharides as demonstrated by a positive reaction with dialysed iron according to Hale (Fig. 8). The presence of these mucopolysaccharides appears to lead to a rupture of many desmosomes (Fig. 5). It is noteworthy that there is no increase in the frequency of gap junctions as observed by Prutkin [16] in keratoacanthomas. The frequency actually decreases slightly from 1 in 7 cells to 1 in 10 cells, a frequency being maintained up to seven days after treatment with retinoids. Intracellularly, in the surviving cells a slight decrease of tonofilaments, polyribosomes and an increase in multivesicular bodies (Fig. 2) and vacuoles containing regular arrays of membraneous material (Fig. 5, inset), (possibly consisting of phospholipids), can be observed, but all these changes are difficult to quantify.

4. *Papillomas treated with Ro* 10-9359, 7 *days after injection*

Light microscopy. The volume of the tumor has shrunk to less than 1% of the original size. There are still a few areas showing hyperplastic epithelium but most parts show an epithelium not much thicker (3–5 nuclear layers) than normal skin. There is also widespread necrosis.

Electron microscopy. Intact cells have at this stage reverted to a practically normal appearance. Only in very few places alterations were detectable in the extracellular space that were so frequent at 48–72 hr. There was, however, one intriguing detail: In many instances mitochondria showed membrane-bound inclusion bodies filled with a homogeneous mass (fatty substances?), 0·1–0·3 μm in diameter. The significance of this observation remains unexplained.

5. *Skin, treated with Ro* 10-9359, 8 *hr to* 3 *days after injection*

No alterations comparable to those seen in papillomas were observed. At a few places only (Fig. 7) were there inclusions and some vacuoles in the extracellular space, the intracellular organelles being undistinguishable from those seen in normal skin.

DISCUSSION

This study was undertaken in order to gain morphological information useful for proving or disproving one of the following hypotheses. The mode of action of retinoids in reversing hyperplasia induced by carcinogens could be due to: (a) a lysosomal labilization leading to autolysis and necrosis, (b) a surfactant action on the plasma membrane resulting in a leaky membrane and consequently in necrosis, (c) an increased formation of gap junctions [16] possibly providing a signal for mitotic arrest, (d) an increased production and secretion of mucopolysaccharides into the extracellular space with an enlargement of this latter. This would lead to a loss of anchorage of the cells and, concomitantly, to a deprivation of nutrients. This hypothesis would be compatible with findings obtained in other systems [21, 22].

Hypothesis (c) can be eliminated easily for two reasons: Firstly, the frequency of gap junctions does not increase in our system during retinoid treatment, but rather decreases. Secondly, it is difficult to see how such junctions could induce necrosis since they are a normal constituent of a variety of normal cells, including epidermal cells.

Hypothesis (a) is in line with many earlier observations in different systems [14]. The activation of the lysosomes in the retinoid treated papillomas, however, is not striking. It is therefore doubtful whether much importance can be ascribed to this mechanism.

Hypothesis (d) is a very attractive one and would explain the formation of necrosis most easily. However, it must be remembered that many necrotic cells are not surrounded by a thick layer of mucopolysaccharides which casts a doubt on the importance of that mechanism.

Hypothesis (b) holds that the surfactant action on the plasma membrane is the cause of necrosis and cell loss. This is certainly the mechanism that is directly and most strikingly supported by our data and probably the most likely mechanism responsible for the effects of retinoid treatment.

It is likely, however, that all the aforementioned mechanisms do occur simultaneously and may act synergistically to produce the dramatic regression of the papillomas. It is important, however, to stress that the systemic treatment with Ro 10-9359 has little or no effects on normal skin. These findings are in contrast to those obtained with topical application of retinoic acid on normal skin [23], where similar phenomena were observed as those described in papillomas in the present study. This is most encouraging in view of the

badly needed specificity of retinoids for potential clinical use. Clinically useful retinoids must show a high degree of specificity, i.e. good anti-tumor activity with little toxic (A-hypervitaminotic) effects. Our study shows that this lies in the realm of possibility.

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Breast Cancer: The Long Latent Interval

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Abstract—*A group of female patients who developed recurrence* 5 *yr or more after their initial treatment for carcinoma of the breast have been studied retrospectively. It is concluded that there is no justification for the concept of an immunological or hormonal control maintaining the cancer cells in a dormant state during the latent interval. The results of this study are consistent with the theory of constant growth rate of the tumours.*

INTRODUCTION

IT IS a well recognized fact that, in a small proportion of patients with breast cancer, there is a long latent interval between primary treatment and the first recurrence. The reasons for this are not obvious at first sight. Two conflicting theories have been proposed: that of slow but constant growth rate [1–5] and that of hormonal or immunological control, maintaining the cancer cells in a dormant state [6–10].

MATERIAL AND METHODS

In this paper, an attempt has been made to identify the correct theory by a retrospective analysis of 139 patients with breast cancer, who had a disease-free period of 5 yr or more. This comprised the total number of such patients registered at the Christie Hospital during the years 1956–1961 inclusive.

This group has been compared with groups of unselected patients with breast cancer. Details of these have been obtained from the literature, with the exception of the group with initial bone recurrence which has been assessed by the author. In addition the author's group of patients having a long latent interval has been subdivided, and the subgroups compared with each other. Also the relationship has been investigated between latent interval and survival after recurrence for those patients who had a long latent interval with initial chest wall recurrence.

Accepted 29 December 1976.

RESULTS

Comparison has been made with respect to age of patients at presentation (Fig. 1) [11], site of tumour in the breast (Table 1) [12], and metastatic pattern (Table 2) [13]. With the exception of a difference in primary metastatic pattern, the groups are similar. The greater incidence of local recurrence in the primary metastatic pattern of the author's group probably resulted from the fact that a smaller percentage of these had received post-operative X-ray therapy to the chest wall and glandular areas.

The duration of the latent interval in the author's group showed a random scatter throughout the age distribution (Fig. 2).

A semi log plot of the survival curve for the whole of the author's group has been compared with that constructed from data published by Campos [14] for 274 unselected patients dying from breast cancer (Fig. 3). It was found that the difference between the slopes is nowhere near statistically significant ($Z = -0.05$). This indicates a similar mortality rate for the two groups of patients.

Survivals in the author's group following initial recurrence on the chest wall have been compared with survivals after initial recurrence at this site in a group of patients with breast cancer having unselected latent intervals (Fig. 4) [13]. Both groups had initial chest wall recurrence alone or coincident recurrence at other sites. There is a statistically significant difference ($P < 0.005$).

Similarly, a comparison has been made between survivals after initial recurrence in bone, in the author's group of patients having a long latent interval, and a group of patients

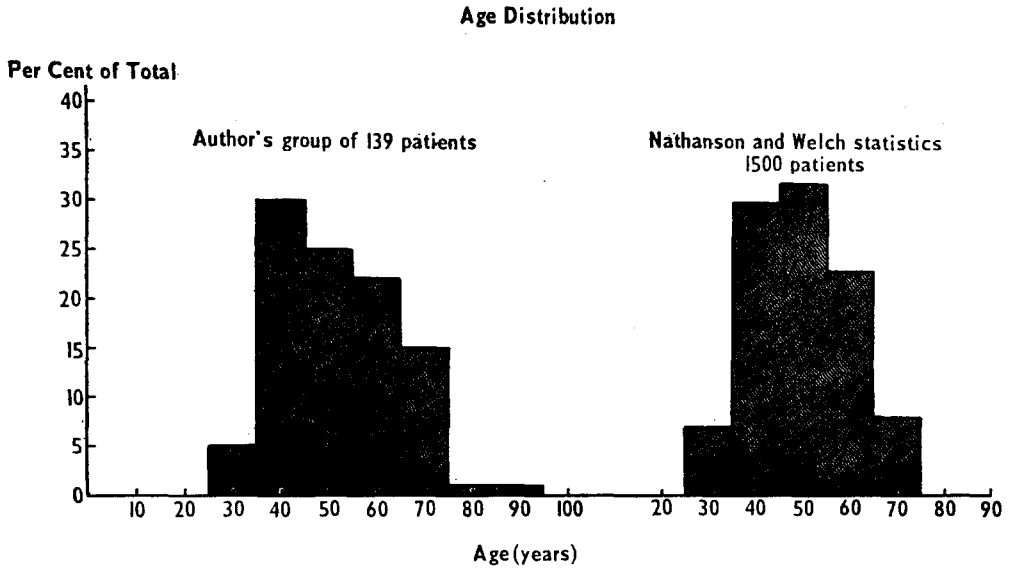

Fig. 1. *Histogram of age distribution compared with that from Nathanson and Welch's statistics.*

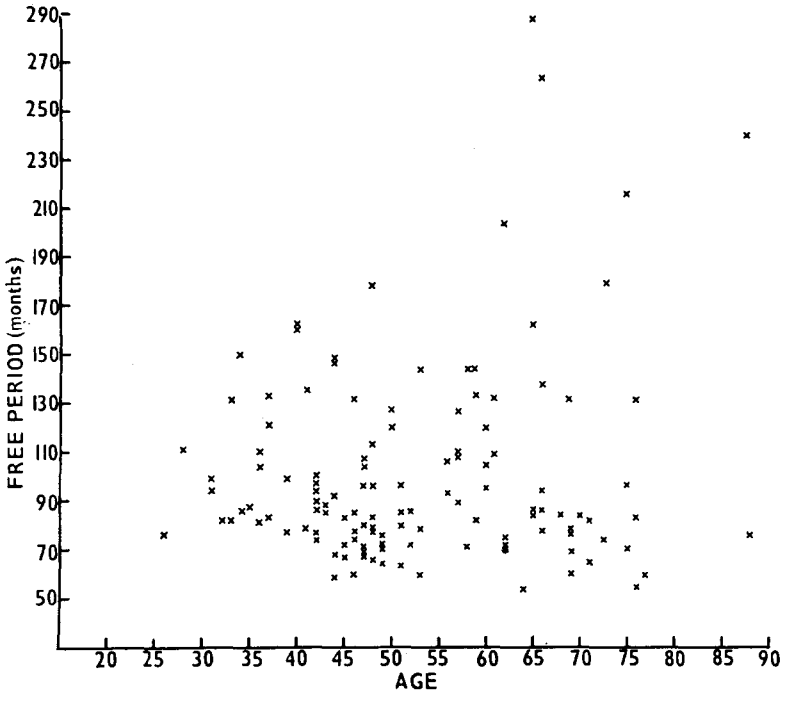

Fig. 2. *Scatter diagram. Duration of latent period plotted against age of patients.*

Table 1. *Site in breast of primary tumour—this group compared with group of Luff*

	This group	Luff, A.P. 1523 patients 1932
U.O.Q.	43%	43.9%
L.O.Q.	15%	16.4%
U.I.Q.	16%	14.1%
L.I.Q.	11%	6.6%
Nipple area	15%	19%

recurring at this site initially, but having unselected latent intervals. There was a statistically significant difference (Table 3) ($P < 0.02$).

Insufficient numbers of patients developed initial recurrences at other sites for useful statistical analysis.

Patients developing initial chest wall recurrence alone, who did not have induction of the menopause or X-ray therapy to the chest wall as part of their initial treatment, have been selected from the author's group of patients

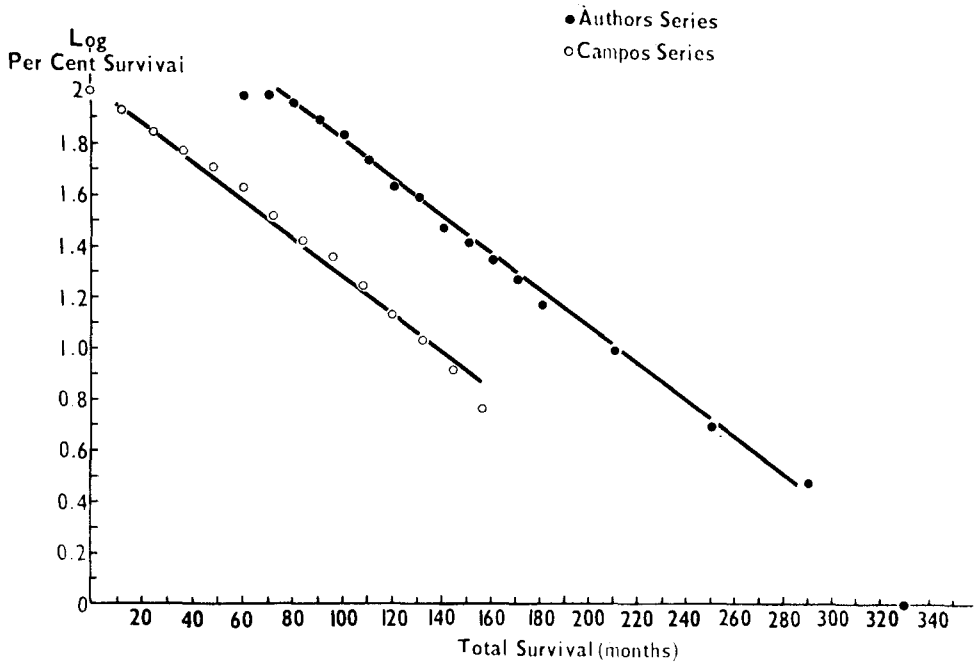

Fig. 3. Comparison of survival curve for all patients in this group with that obtained from Campos's figures.

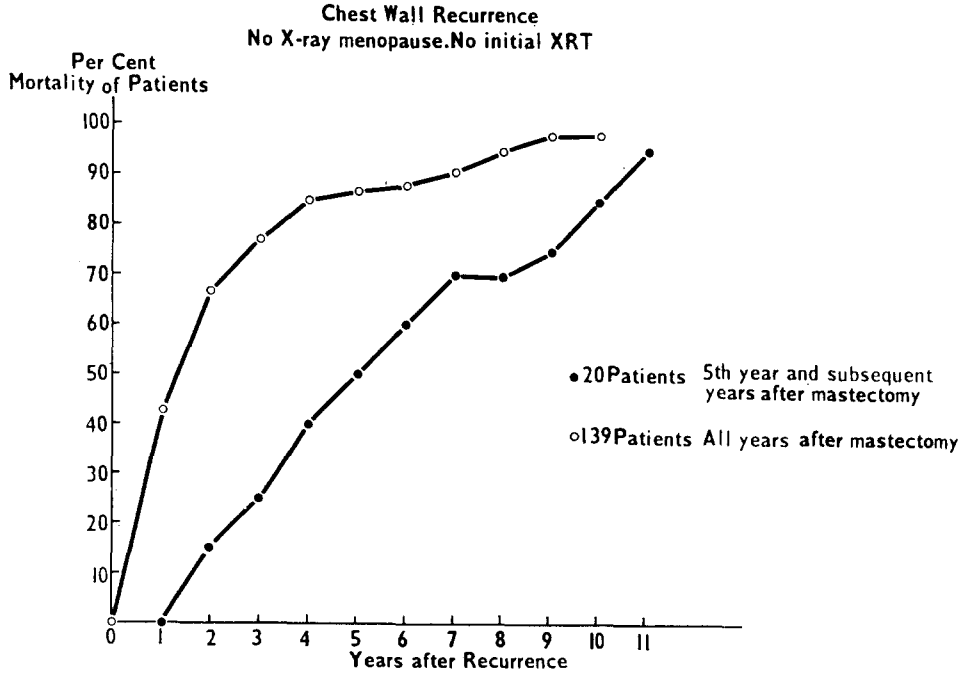

Fig. 4. Chest wall recurrence. Comparison of survival curves obtained for this group and Jackson's group.

having a long latent interval. For these patients the latent interval has been plotted against survival after recurrence. The correlation was highly significant ($P < 0.001$). However, as there were few patients with extreme values of duration of latent period 8 patients were added. These constituted the sum total of patients first registered during the years 1951–1955 inclusive at the Christie Hospital who fulfilled the above criteria for inclusion into the correlation. There is now no doubt concerning the highly significant correlation ($P < 0.001$) (Fig. 5).

From the author's group of patients having a long latent interval, subgroups have been selected by virtue of histologically involved axillary nodes, muscle fixation of primary tumour, poor differentiation or anaplasia and palpable but histologically negative axillary nodes or reactive hyperplasia. It was found that

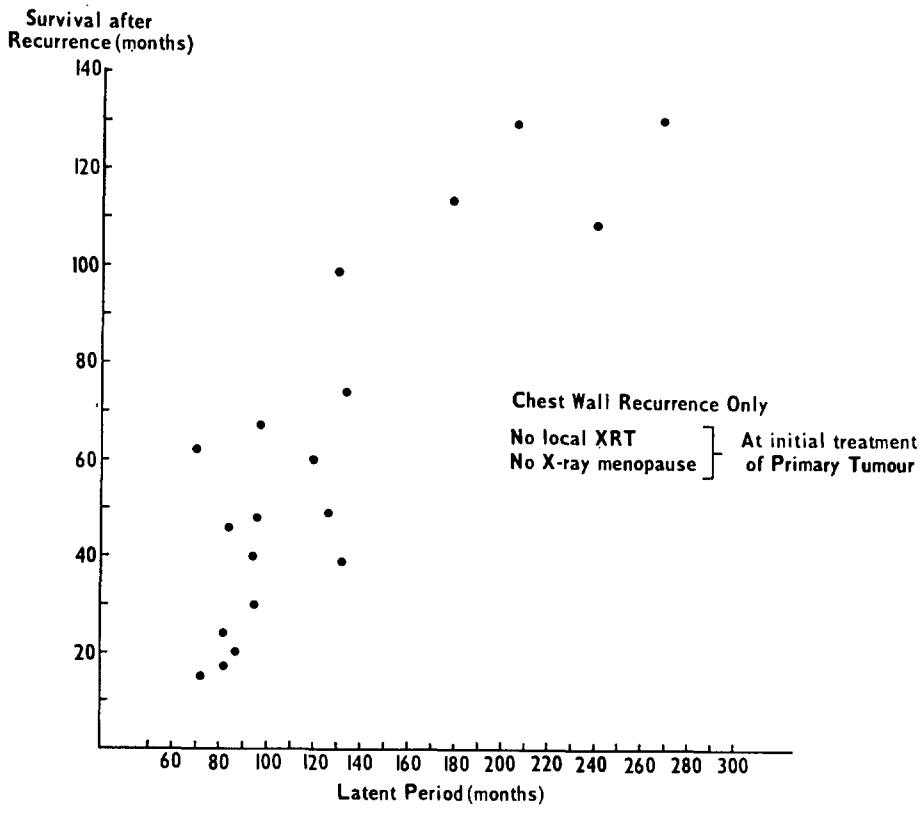

Fig. 5. *Scatter diagram. Latent period plotted against survival times following recurrence.*

Table 2. *Metastatic pattern—this group compared with Jackson's group*

	This group	S.M.J. thesis 1461 patients
Primary metastatic pattern		
Lung	17%	21%
Liver	4%	16%
Bone	19.3%	26%
Local	45%	34%
Subsequent metastatic pattern following local recurrence		
Lung	34%	35%
Bone	36%	28%
Liver	16%	18%

there is no significant difference in survivals from subgroups not displaying these features (Tables 4–6).

DISCUSSION

The patients in the author's group received a variety of treatments, both for the primary tumour and for recurrent disease. However, different modes of therapy seem to make little difference to the survival figures for patients with breast cancer [13, 15–18]. It is therefore reasonable to compare survivals in the author's group with survivals in unselected groups of patients with breast cancer. However, the ratio of latent interval to survival after recurrence may be altered by induction of the menopause or by local X-ray therapy to the chest wall, given as part of the initial treatment. Patients who received these treatments have been excluded from analysis when survival after recurrence is considered.

It has been demonstrated in this paper that the author's group of 139 patients, with a long latent interval, is similar to an unselected population of patients with breast cancer in all features investigated, with the exception of a longer survival after initial recurrence in bone and chest wall. When a tumour is clinically apparent it grows, in most cases, for most of the time in an exponential manner [1, 3, 4]. Also tumour growth rate is proportional to survival times [19]. Therefore, patients with breast cancer having long latent intervals are those with tumours which when clinically apparent exhibit a slow constant growth rate. It would seem reasonable to assume a similar slow constant growth rate giving rise to a long latent interval and it is unnecessary to postulate hormonal or immunological control to account for this phenomenon. This contention receives support from the highly significant correlation when latent interval is plotted against survival after recurrence on chest wall.

Table 3. Solitary bone recurrence—survival of this group compared with author's random group. No X-ray menopause as part of initial treatment. None alive

	Survival after recurrence	Dead after		No.
		6 months	12 months	
Author's group Long latent intervals	(2–108) Median 21 months	16%	31%	13
Author's group Unselected latent intervals	(0–106) Median 8 months	41%	60%	80

Table 4. Axillary nodes—comparison of survival times for negative nodes, positive nodes, and palpable nodes negative or nodes showing reactive hyperplasia

	No.	Survival after original treatment	Survival after recurrence
Nodes negative histologically	45	59–292 Median 107 months	0–130 Median 17
Nodes positive histollogically	48	71–213 Median 111.5 months	1–93 Median 15
Palpable nodes negative or reactive hyperplasia	9	78–147 Median 103 months	0–55 Median 11

Table 5. Muscle fixation—comparison of survival times for tumour fixed and not fixed to muscle

	No.	Survival after original treatment	Survival after recurrence
No fixation	41	49–334 Median 103 months	0–130 Median 18
Fixation present	16	70–139 Median 106.5 months	2–55 Median 10.5

Table 6. Histology of original tumour—survival times

	Survival after original treatment	Survival after recurrence
13 patients Poorly differentiated or anaplastic	88–179 Median 94 months	0–59 Median 18 months
126 patients Grade not stated	59–179 Median 106 months	0–144 Median 21.5 months

One of the main objections to the theory of constant growth rate is that some patients who present with apparently advanced or aggressive cancers have long disease free intervals following their initial treatment [13]. Examples of these tumours are those with muscle fixation, or where there is histological evidence of axillary node involvement, or where the tumour was poorly differentiated or anaplastic. It has been shown in this paper that survivals in groups of patients with these tumours is similar to those of patients having tumours without these features.

It has been suggested that palpable, but

histologically negative axillary nodes, or reactive hyperplasia is an indication of enhanced immunological host activity [20]. However, an analysis of the author's group of patients has shown that the survival times of patients having these features is similar to cases not exhibiting these phenomena.

Since survival is proportional to tumour growth rate, these results indicate that the whole of the author's group of patients is homogeneous in respect of tumour growth rate, and there is therefore no evidence of a separate immunological or hormonal control in the subgroups considered.

It is a clinically apparent fact, that in a few patients with breast cancer, endocrine manipulations may lead to prolonged remissions. However, long term remissions tend to occur in patients who have had long latent intervals [21]. These are therefore patients with slowly growing tumours. The induction of the menopause by irradiation, performed shortly after mastectomy delays the onset of clinical recurrence in a few patients for short periods, but does not significantly alter survival [22–24]. It is therefore suggested that remissions occur as a result of minor changes in tumour doubling time and that very little deviation from the exponential growth rate would be expected from alterations in the hormonal environment of the tumour during the latent intervals.

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Effects of Inducers and Inhibitors of the Benzo(a)pyrene Hydroxylase of Isolated Rat Liver Nuclei and Nuclear Envelopes on the Binding of Benzo(a)pyrene to DNA*

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Abstract—*Rat liver nuclei obtained from rats pretreated with different inducers of mixed function oxidases were incubated with* ^{3}*H-benzo(a)pyrene* (^{3}*H-BP*). *The binding of* ^{3}*H-BP to the DNA of these nuclei was found to increase only after induction by* 3-*methylcholanthrene* (3*MC*), *but not after pretreatment of rats with phenobarbital* (*PB*) *of pregnenolone* 16α-*carbonitrile.*

7, 8-*Benzoflavone* (7, 8-*BF*), *a known inhibitor of aryl hydrocarbon hydroxylase, inhibited to about* 80% *the binding of* ^{3}*H-BP to DNA if the nuclei were obtained from* 3*MC-pretreated rats, but not if they were from PB-pretreated or control rats.*

When 1, 1, 1-*trichoropropene*-2, 3-*oxide* (*TCPO*), *an inhibitor of BP epoxide hydratase, was added to the nuclei, the binding of* ^{3}*H-BP to DNA increased in the nuclei of both untreated and treated rats. Microsomes obtained from* 3*MC-pretreated rats when added to the nuclei increased the binding* 2–5 *times. A slight decrease in binding occurred when control microsomes were incubated with nuclei from* 3*MC-pretreated rats as compared to the same incubation without microsomes.*

When these experiments were carried out with denuded nuclei, the addition of nuclear membranes to the medium increased the binding only when the membranes were from 3*MC-pretreated rats. The effect of added* 7, 8-*BF or TCPO to the medium was similar to that observed with intact nuclei. These latter results show that the enzyme activity which activated BP to species that bind to DNA is localized in the nuclear envelope.*

INTRODUCTION

BENZO(A)PYRENE (BP), one of the most common carcinogenic hydrocarbons, is metabolized by aryl hydrocarbon hydroxylase (AHH), the microsomal NADPH-dependent mixed-function oxygenase which contains cytochromes P-448 or P-450 [1], and BP epoxide hydratase (EH) [2]. The action of the mixed-function oxygenases and the hydratase results in the conversion of BP to oxides, phenols, dihydrodiols, diolepoxides and quinones [1, 3].

Although the metabolic activation of BP is believed to take place chiefly in microsomes, such activation has recently been demonstrated with nuclei [4–7]. Increased binding of BP to nuclear DNA was observed in rat liver nuclei from 3-methylcholanthrene (3MC)-pretreated rats [4, 6, 8, 9]. The binding of ^{3}H-BP to DNA in isolated rat liver nuclei was very similar in nature to that which occurred when BP was added to calf thymus DNA in the presence of microsomes [5].

At least two forms of the BP hydroxylating enzyme system (AHH), which can be differentially induced *in vivo* by administration of phenobarbital (PB) or 3MC, have been found in rat tissues [10]. One, therefore, ought to be able to increase or decrease the ability of a potential carcinogen to bind to DNA by inhibiting or stimulating these drug metabolizing enzymes or by adding agents which

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selectively compete with DNA as a substrate for these carcinogens. 7, 8-Benzoflavone (7, 8-BF), an inhibitor of AHH, markedly inhibits this enzyme in hepatic microsomes from 3MC-pretreated rats but not from untreated rats [10]. 1, 1, 1-Trichloropropene-2, 3-oxide (TCPO), an inhibitor of hepatic epoxide hydratase (EH), increase BP binding to exogenous DNA in the presence of liver microsomes [3].

This paper describes studies on the effect of inducers and inhibitiors of AHH and EH on the binding of BP to DNA in rat liver nuclei *in vitro*.

MATERIAL AND METHODS

Chemicals

^{3}H-labelled BP (specific activity, 25 Ci/mmole) was purchased from the Radiochemical Centre, Amersham, England, and purified on 0.25-mm thick silica gel (Merck, Germany) with benzene. All other chemicals used were as previously noted [5].

Treatment of animals and preparation of nuclei and nuclear membranes

Sodium phenobarbital (0.1%) was placed in the drinking water of 120- to 150-g male Wistar rats (Wag strain) for 10 days; a micronized suspension of pregnenolone 16α-carbonitrile (PCN) in 2 ml water with a trace of Tween 80 was given *per os* to another group (50 mg/kg). The compound was administered at 8-hr intervals (twice daily for 2 days and once on the 3rd day); 3-methylcholanthrene in 0.5 ml corn oil was injected i.p. (25 mg/kg) daily for two days. Control animals received corn oil only. Decapitation was preceded by a 24-hr fast. Nuclei from rat liver were isolated as previously described [5] and were used immediately without storage. All preparations were routinely examined by light and electron microscopy. The nuclei appeared to be normal, intact and essentially free of contaminations from organelles such as microsomes [5]. The nuclear membranes were prepared according to Kay [11] using DNAse I.

Binding of ^{3}H-BP to nuclear DNA

The nuclear pellet was resuspended in 0.05 M Tris–HCl buffer (ph 7.5) containing 0.003 M $MgCl_2$ and NADPH (1.3×10^{-4}M). In 1 ml of medium were suspended the nuclei from about 2 g of liver, liver microsomes (1 or 2 mg of protein) and ^{3}H-BP at 80 nmole in 50 μl acetone. 7,8-BF was added at a concentration of 10^{-4}M and TCPO at 5×10^{-3}M. For the blanks, we used an incubation medium without nuclei. The mixture was incubated for 15 min at 37°C. Thereafter, the incubation medium was chilled to 4°C and the nuclei were sedimented at 1000 $\times$ **g** for 10 min. The nuclear pellet was suspended in 0.05 M Tris–HCl buffer, made 6% with sodium p-aminosalicylic acid (PAS) and 1% with sodium dodecylsulfate (SDS) and the solution was extracted with a phenol mixture [5]. The DNA was precipitated with two volumes of ethanol in the presence of 2% CH_3COONa, then washed with ethanol. The DNA was redissolved in 0.01 M phosphate buffer (pH 7.0) and the solution was incubated with 100 μm/ml of preheated RNAse (80°C for 10 min) at 37°C for 30 min and subsequently for 1 hr with 100 μg/ml preheated pronase (DNAse-free). The solution was adjusted to 1 N $NaClO_4$ and washed with $CHCl_3$- isoamyl-alcohol (24:1). The aqueous phase was washed with ether and the DNA was precipitated as above, washed with ethanol and ether and dried. The DNA was dissolved in water at a concentration of 0.05–0.08 mg/ml. One ml was added to 10 ml of a Triton X100-based scintillant and the radioactivity was measured in an Intertechnique Counter model LS30 with a 29% efficiency for ^{3}H. Samples (0.5 ml) were taken before and after incubation for the AHH enzyme assay as described elsewhere [5].

RESULTS

Effect of inducers on AHH activity and on the binding of ^{3}H-BP to nuclear DNA

The results show 10–16 times more enzymatic activity in the nuclei from MC-treated rats than in those from the control, PB- and PCN-pretreated animals (Table 1). The uninduced nuclei, however, activated BP which bound at low levels to DNA (0.8 μmole BP/mole P-DNA). The binding levels we observed in uninduced nuclei were about 2 times lower than those found by Rogan *et al.* using ^{14}C-BP [6, 8]. PB and PCN did not increase the binding of BP to DNA, whereas 3MC pretreatment increased the binding to the nuclei of the controls about 5 times. These results show that the increased binding level responded only to induction by MC which specifically induces cytochrome P-448 enzyme activity [1, 12] and did not respond after pretreatment of rats with PB or PCN which induce cytochrome P-450.

Table 1. Effect of various inducers of microsomal mixed-function oxidases on nuclear membrane-mediated binding of ^{3}H-BP to DNA of rat liver nuclei. Twenty-four hours prior to sacrifice, rats were given injections of 25 mg of 3-methylcholanthrene (3MC) in 0.5 ml corn oil; phenobarbital (PB) and pregnenolone 16α-carbonitrile (PCN) were administered as described in Materials and Methods. The liver nuclei were incubated for 15 min at 37°C and aliquots (0.5 ml) were removed for AHH determinations. The DNA of the nuclei was isolated as described in Material and Methods

Liver nuclei	AHH*	Enzymatic activity ratio to controls	Binding of ^{3}H-BP to DNA†	Binding ratio to controls
Control rats	100	1.0	0.8 ± 0.2	1.0
PB-treated rats	140	1.4	0.9 ± 0.4	1.1
PCN-treated rats	152	1.5	1.0 ± 0.5	1.3
3MC-treated rats	1604	16.0	3.8 ± 1.5	4.8

*The mean specific activity for the uninduced (control) rat liver nuclei based on more than three separate experiments is equal to 142 pmoles/30 min/mg protein.
†The binding of ^{3}H BP is expressed in μmoles BP/mole P-DNA.

Effect of inhibitors on the AHH and EH activities and on the binding of ^{3}H-BP to nuclear DNA

7, 8-Benzoflavone, a known inhibitor of AHH, exhibited a contrasting effect on the binding of carcinogen when added to the incubation medium at a concentration of 10^{-4}M (Table 2). With control nuclei and nuclei from BP-treated rats, 7, 8-BF had no effect on AHH and on the binding of ^{3}H-BP to DNA. With nuclei from 3MC-pretreated rats, about 80% inhibition of AHH was observed, and BP binding to DNA was inhibited to a level of 0.9 μmole of BP per mole P-DNA which correspond to that observed in nuclei from control and PB-pretreated rats.

When TCPO, a potent inhibitor of BP epoxide hydratase, was added to the nuclei at high concentrations (5×10^{-3}M), the AHH activity (mixed function oxidases) was inhibited. This was supposed to reduce epoxide formation which would reduce binding. However, the binding of ^{3}H-BP to DNA was increased in the nuclei of both untreated and treated rats

Table 2. Effect of 7, 8-BF and TCPO on the binding of benzo(a)pyrene to the DNA of rat liver nuclei. The table represents the average of values obtained in at least three separate experiments. The quantity of protein was 2.5–3 mg/ml of medium ^{3}H-BP (specific activity 1.25–2.5 Ci/m-mole) was incubated with the nuclei in the presence of 10^{-4}M 7, 8-BF and 5×10^{-3}M TCPO

Treatment of animals	Additions	Binding of ^{3}H-BP to DNA (μmole/mole-P)*	(% of controls)
Control	None	1.1	100
	7, 8-BF	1.2	109
	TCPO	2.8	254
Phenobarbital	None	1.0	100
	7, 8-BF	1.0	100
	TCPO	1.9	190
Methylcholanthrene	None	4.6	100
	7, 8-BF	0.9	20
	TCPO	7.4	161

*1 Unit = The fluorescence equivalent to 1 pmole 3-OH-BP formed in 30 min/mg of protein.

(Table 2) which must mean that the essential action of TCPO is inhibition of epoxide hydratase.

Effect of added liver microsomes on BP binding to DNA of liver nuclei

Liver microsomes at two different concentrations (1 and 2 mg of protein per ml) from untreated and 3MC-pretreated rats were added to the purified liver nuclei obtained from untreated and 3MC-pretreated animals (protein concentration 1–2 mg/ml) (Table 3). Slight inhibition (~ 25%) was observed only when microsomes from untreated (control) rats were added to the nuclei from 3MC-pretreated animals. A 5–8-fold greater binding occurred when microsomes from 3MC-pretreated animals were added to control nuclei. A lesser increase in binding (~ 2-fold) was observed when both microsomes and nuclei were obtained from 3MC-pretreated rats.

Table 3. The effect of added liver microsomes on ^{3}H-BP binding to the DNA of purified rat liver nuclei. ^{3}H-BP was incubated with nuclei from 3-methylcholanthrene (3MC)-pretreated rats or control nuclei in the presence of microsomes obtained from the liver of corn oil-treated (Control) or 3MC-pretreated rats. The ratio of binding was determined by dividing the binding results of each group by the results obtained without microsomes

Nuclei	Added microsomes	Protein of microsomes (mg/ml)	^{3}H-BP binding to DNA (μmole/mole-P)	Ratio of binding with to without microsomes
Control	None	0	1.1	1.0
	Control	1	1.2	1.1
	Control	2	1.0	0.9
	3MC	1	5.6	5.1
	3MC	2	9.2	8.4
3MC	None	0	5.5	1.0
	Control	1	3.9	0.7
	Control	2	4.2	0.8
	3MC	1	10.6	1.9
	3MC	2	12.1	2.2

Experiments with Triton X-100

The addition of Triton X-100 at concentrations between 0.5 and 2% to rat liver nuclei leads to complete inhibition of AHH activity in the envelope-denuded nuclei and of the binding of ^{3}H-BP to DNA in all three nuclear preparations from untreated animals and animals pretreated with PB and 3MC (Fig. 1). Treatment with Triton X-100 removed the nuclear envelope and the associated ribosomes leaving the nuclear contents morphologically intact. These results show that enzymes present in the nuclear envelope are capable of activating benzo(a)pyrene to species that react with DNA.

Addition of nuclear membranes to denuded nuclei and the effect of inhibitors on enzyme activity

The addition of nuclear membranes at concentrations of 0.4–0.6 mg of protein per ml of medium leads to the metabolism of BP and subsequently to its binding to DNA (Table 4). The binding increased only when nuclear membranes of liver nuclei from MC-pretreated rats were added to the medium. When 7, 8-BF (10^{-4}M) or TCPO (5×10^{-3}M) were added to the medium containing denuded nuclei and nuclear membranes from 3MC-pretreated rats, the effect was similar to that observed with intact nuclei and microsomes from 3MC-pretreated rats (Table 5). 7, 8-BF inhibited to about 80% the binding of ^{3}H-BP to DNA while TCPO increased it. These results show that the enzyme activity which activated BP to species that bind to DNA is localized in the nuclear envelope.

DISCUSSION

There are recent indications that differences exist between nuclei and microsomes for the metabolic activation of hydrocarbons and their

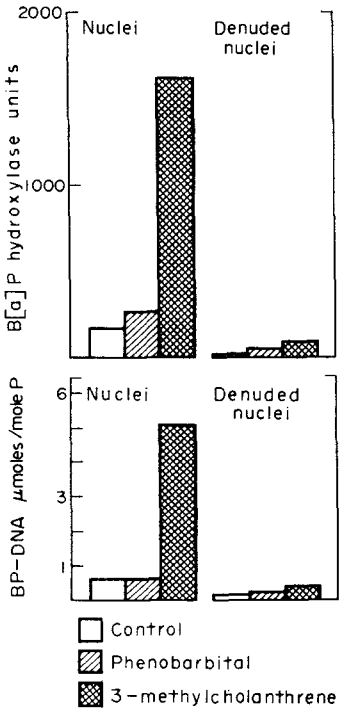

Fig 1. *The effect of Triton X-100 on the BP hydroxylase activity (AHH) and BP binding to DNA in rat liver nuclei.*

Denuded rat liver nuclei were obtained by adding 0.5% *Triton X-100 to isolated liver nuclei. The BP hydroxylase activity and BP binding to DNA in both denuded and intact nuclei were determined as described in Material and Methods.*

binding to DNA [6, 8]. In a previous study [5], we observed a similarity between BP-DNA binding catalyzed by the liver nuclei and liver microsomes from 3MC-pretreated rats.

The levels and induction of nuclear AHH activity that we observed were similar to those reported by others [6, 8, 13] for nuclei from control rats and those from 3MC- or PB-pretreated rats. Again 3MC had a much greater inductive effect than PB, which did not increase the level of nuclear AHH. The ratio of nuclear to microsomal AHH was two to four times higher in 3MC-pretreated rats than in PB-pretreated rats [5]. The effect of PB induction was greatest on the level of nuclear cytochrome P-450 [8].

The amount of hydrocarbon bound to DNA in nuclei from untreated rats was generally low (Table 1). We observed the same with nuclei from PB-pretreated rats while others [8] found that PB doubled the binding; 3MC induction increased the level of binding for all hydrocarbons studied. These data suggest that the potent carcinogen 3MC preferentially induces nuclear enzymes, possibly increasing the binding of hydrocarbons to DNA with the likelihood of tumor initiation.

The addition of TCPO increased the binding level of ^{3}H-BP to DNA in control nuclei and in

Table 4. *Binding of ^{3}H-BP to DNA in denuded rat liver nuclei in the presence of nuclear membranes from treated and untreated rats. The nuclear membranes from controls, phenobarbital (PB)-pretreated or 3-methylcholanthrene (3MC)-pretreated rats were added at a concentration of* 0.5–0.7 *mg/ml of protein to the incubation medium containing the denuded nuclei. Figures represent the mean of triplicate determinations*

Nuclear membranes	^{3}H-BP binding to DNA	
	(μmole/mole-P)	(% of Controls)
None	0	0
Control	1.4	100
PB-treated rats	2.0	143
3MC-treated rats	8.2	586

Table 5. *The effect of* 7, 8-*BF and TCPO on ^{3}H-BP binding to DNA in denuded nuclei in the presence of nuclear membranes (NM) from 3-methylcholanthrene-pretreated rats.* 7, 8-*BF was added at a concentration of* 10^{-4} *and TCPO at* $5 \times 10^{-3} M$

Addition to denuded nuclei	^{3}H-BP binding to DNA in denuded nuclei	
	(μmole/mole-P)	(% of controls)
None	0	0
NM	5.4	100
NM+7, 8-BF	0.9	17
NM+TCPO	7.6	140

nuclei from pretreated animals. TCPO at the concentration used [3] is known to inhibit completely the formation of BP diols and the AHH activity. At this concentration TCPO may also interfere with BP epoxide hydration. The increased ^{3}H-BP binding contributed to the increased quantity of BP oxides and perhaps BP dioxides whose hydrations were inhibited by TCPO. However, Jernström *et al.* [14], using a lower concentration of TCPO, did not observe inhibition of the formation of BP phenols nor of the binding to DNA. 7, 8-BF reduced the binding of ^{3}H-BP to DNA in the liver nuclei from 3MC-pretreated rats, but had no effect on the binding in nuclei from control and PB-pretreated rats. With nuclei from control and PB-pretreated rats, 7, 8-BF did not inhibit the formation of the BP phenol fraction (unpublished results), while with nuclei from 3MC-pretreated rats, 7, 8-BF strongly inhibited phenol formation to an extent similar to that observed with microsomes from 3MC-pretreated rats [10].

The washing of nuclei with Triton X-100 has been shown to remove the nuclear envelope [7, 8]. The simultaneous removal of binding and AHH activities clearly shows that the binding activity as well as the enzyme are associated with the nuclear envelope. The effect of 7, 8-BF and TCPO on the binding activity of nuclear membranes from the nuclei of 3MC-pretreated rats (Table 5) was similar to that observed with all nuclei (Table 2) and with the microsomes from 3MC-pretreated animals [3, 10]. This suggested that the 3MC-induced enzyme involved in the nuclei-dependent BP binding has the same response as that involved in the microsome-dependent BP binding to DNA [3, 15]. We observed a significant increase in the binding of ^{3}H-BP to DNA when the microsomes used were from 3MC-pretreated rats, and slight inhibition with microsomes from untreated animals only when they were incubated with nuclei from 3MC-pretreated rats. These results are in agreement with the results recently published by Pezzuto *et al.* [16] and Jernström *et al.* [14], but are in contrast with the findings of Vaught and Bresnick [9] who recently reported a reduction of BP binding to nuclear DNA when microsomes from untreated rats were added to the nuclear incubation systems, and no effect when microsomes from 3MC-pretreated rats were used. This difference may be due to the low concentration of ^{3}H-BP used in their study. The *in vitro* system used in this study (nuclei and microsomes) closely approximates the *in situ* condition. However, further studies are needed to clarify the role of microsomes in the binding of ^{3}H-BP to DNA in rat liver nuclei.

The manner in which BP is activated in the cell seems to be well established [17, 18]. Recent work shows differences between nuclei and microsomes in the metabolic activation of hydrocarbons and their binding to DNA [6]. We observed a similarity between BP-DNA binding catalyzed by the liver nuclei and liver microsomes from 3MC-pretreated rats [5].

Nagata *et al.* [19] have reported that the nucleus has an enzymatic activity capable of converting BP to the 6-oxo-BP radical. It has also been proposed [8] that cytochrome P-450 rather than other enzymes is involved in the hydrocarbon activation, the one-electron oxidation being the mechanism by which hydrocarbons bind to cellular components. Such oxidations of the substrate would produce a reactive intermediate radical cation capable of binding to cellular nucleophiles [8].

This study has shown that the activity observed with liver nuclei is identical with that of the microsomes from the same animals, and that it is possible to alter *in vitro* the binding of BP to nuclear DNA by adding inhibitors of AHH and EH.

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Highly Cytotoxic Antisera Obtained in W/Fu Rats against a Syngeneic Gross Virus Induced Lymphoma*

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Abstract—*Antisera raised in W/Fu rats against the syngeneic (C58NT)D lymphoma induced by the Gross agent may provide specific antibodies for passive immunotherapy of Gross virus-induced murine leukaemia. Searching for high antibody activity towards tumour cells, which is highly desirable for passive immunotherapy, eight immunization schedules were assayed in 68 W/Fu rats: groups of five to thirteen adult rats received subcutaneously an injection of 100–700 × 10^6 viable (C58NT)D cells, followed after an interval of three to eight weeks, by weekly subcutaneous injections of 50–250 × 10^6 viable cells. The injection of 400 × 10^6 tumour cells followed four weeks later by five weekly injections of 200 × 10^6 cells gives highly cytotoxic antisera. However, kinetic studies of the antibody response show that if near maximal titers may be reached twenty days after a single inoculation, highly cytotoxic antisera were only reliably obtained after three booster injections. In repeatedly inoculated rats, blood could be drawn several times from the same animal, between the fourth and the twentieth day after the last injection without loss in cytotoxic activity.*

INTRODUCTION

ANTISERA raised in W/Fu rats against the syngeneic (C58NT)D lymphoma induced by the Gross agent have provided a new insight into the definition of cell surface antigens associated with murine leukaemia viruses [1]. These antisera, presently used as typing sera, appear to recognize at least three types of cell surface antigens induced by the Gross agent (GCSAa, GCSAb, G_{IX}) [1, 2], by other murine leukaemia viruses (GCSAb) [1, 3] or even expressed on some normal cells [4]. These antisera have also been used for passive immunotherapy of Gross virus induced murine leukaemia [5]. It is highly desirable that antisera used for passive immunotherapy should have a high activity towards tumour cells. Up to now, three immunization schedules have been used for the production of antisera to Gross virus associated cell surface antigens [1, 5, 6]; however, the cytotoxic titers of the antisera obtained were usually rather moderate and only a few animals occasionally produced high activity antibodies.

We tried different hyperimmunization schedules of adult W/Fu rats with (C58NT)D cells, searching for high antibody activity and reproducibility. The results of these attempts are presented here. The kinetics of the antibody response was followed on some rats.

MATERIAL AND METHODS

Animals

W/Fu rats were originally provided by Dr. J. C. Leclerc (INSERM, Hôpital Cochin, PARIS) and bred in the laboratory; 2.5–5-month-old rats were used for hyperimmunization. W/Fu rats used for the study of the kinetics of antibody production were purchased from Iffa-Credo (Les Oncins, St-Germain-sur-l'Arbresle, France). C57Bl/RhoIco mice were obtained from Iffa-Credo and bred in the laboratory.

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Tumour cells

The W/Fu (C58NT)D lymphoma, a syngeneic Gross virus induced lymphoma of W/Fu rat [1] kindly provided by Dr. R. B. Herbermann (National Cancer Institute, Bethesda, U.S.A.) was used. This subline behaves differently from the French-NTD [7]: subcutaneous inoculation of $400–700 \times 10^6$ cells in adult rats produces a tumour which regresses in nearly all rats within 3–4 weeks, progressive growth being an extremely rare event. The lymphoma is serially transplanted as an ascites by intraperitoneal inoculation of 20×10^6 viable cells into weanling W/Fu rats. Cells used for immunization were harvested from the animals 7 days after transplantation, and washed three times in phosphate buffered saline pH 7.2 before use.

E♂G2 lymphoma, a Gross virus induced lymphoma of C57B1/6 mice [8] originally provided by Dr. E. A. Boyse (Sloan Kettering Institute, New-York, U.S.A.) was obtained from the Institut de Cancérologie et d'Immunogénétique. It is transplanted weekly into 6–7-week-old male C57B1/6 mice, by intraperitoneal injection of 5×10^6 viable tumour cells obtained by mincing the spleen. E♂G2 cells used as target in cytotoxicity assays were washed three times and finally suspended in Hank's balanced salt solution.

Antisera

Antisera to (C58NT)D lymphoma were raised in syngeneic adult W/Fu rats by subcutaneous injections of viable (C58NT)D cells. Eight different schedules were used; briefly, a first injection of a variable number of viable (C58NT)D cells was given on day 0 and followed 3–8 weeks later by several weekly injections of different numbers of viable tumour cells. The number of cells used, the number of booster injections and the duration of the interval between the first and the second injections are detailed in Table 1. Usually small tumours grow after the first injection but spontaneously regress within 3–4 weeks and sometime after the third or fourth injection with rapid regression in 8–10 days. Sera were collected by cardiac puncture under ether anesthesia 8–12 days after the last injection. For the kinetic study of antibody production, blood was collected from the tail.

Cytotoxicity test

The test has been previously described [9]. Rabbit complement, individually preselected for absence of natural cytotoxic activity against normal mouse cells, was used. The titer of an antiserum was expressed as the last dilution giving a cytotoxicity index [9] equal to 0.50. For each group of rats the geometric mean of the cytotoxicity titers of the antisera was calculated: $\text{gmt} = \sqrt[n]{t_1 \times t_2 \times \ldots t_n}$.

RESULTS

Comparison of the different immunization schedules

The cytotoxic activities against E♂G2 cells of the individual antisera obtained in the eight groups of rats are shown on Fig. 1. From these data it can be seen that four parameters appear to play a major role in the determination of the best immunization schedule: the number of (C58NT)D cells used for the first injection, the number of cells used for the booster injections, the time interval between the first and the

Table 1. *Immunization schedules of adults W/Fu rats by* (C58*NT*)*D lymphoma cells*

Group No.	Number of animals	Number of cells for 1st injection ($\times 10^6$)	Interval of time between 1st and 2nd injection (weeks)	Number of viable cells for weekly booster injections ($\times 10^6$)	Number of weekly booster injections
I	8	100	8	50	6
II	7	150	5	200	7
III	5	700	4	250	9
IV	7	450	8	250	9
V	6	300	3	150	7
VI	10	400	4	150	8
VII	13	400	4	200	6*
VIII	12	400	4	200	5

*The last injection was given two weeks after the fifth booster injection.

second injections and the number of booster injections.

The number of cells initially injected does not appear to be critical, but the use of 150×10^6 (group II, gmt = 540) or 400×10^6 viable cells (groups VI and VII, gmt = 500 and 890 respectively) yields better results than 700×10^6 cells (group III) which only gives a mean titer of 170.

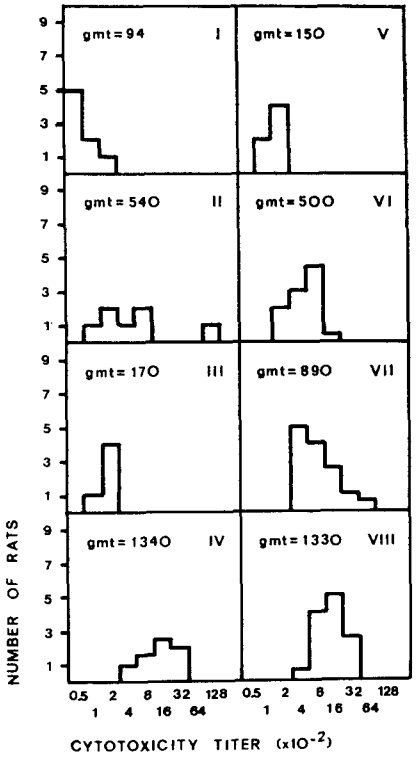

Fig. 1. Cytotoxic activity to E♂G2 lymphoma cells of W/Fu anti (C58NT)D rat sera. Distribution of individual cytotoxicity titers and geometric mean titer (gmt) are indicated for each group of immunized rats (see protocol on Table 1).

A time interval of 4 weeks or more between the first and the second injections yielded high activity antisera (groups IV, VI, VII and VIII). Poor results were obtained when a 3 weeks interval was used (group V, gmt = 150).

Booster injections of 50×10^6 (C58NT)D cells were found to be inefficient (group I, gmt = 94) and higher number of cells were required to produce antisera with high cytotoxicity titers: in group IV a mean titer of 1340 was obtained with booster injections of 250×10^6 cells.

The total number of injections also appears to be an important parameter in the obtention of high activity antisera. Animals in groups VI, VII and VIII received the same initial injection of 400×10^6 (C58NT)D cells, and, after a 4 weeks interval, booster injections of $150–200 \times 10^6$ cells. Antisera obtained in group VIII, after 6 injections, showed a mean titer of 1330, while antisera obtained after 9 injections in group VI showed a lesser activity (gmt = 500). Animals in group VII were immunized identically to animals in group VIII, but they received 2 weeks after the sixth injection an extra booster injection: the cytotoxic activity of antisera obtained in this group of rats was somewhat lesser (gmt = 890) than that of antisera raised in group VIII.

Kinetic studies of the cytotoxic antibody response during immunization

Five 10-week-old W/Fu rats were immunized identically to animals in group VIII (Table 1). Injections were given on days 0, 28, 35, 42, 49 and 56; blood was drawn from the tail of each rat during immunization at days 1, 7, 13, 20, 27, 35, 41, 48, 55, 60, 64, 67, 72, 76 and 79. The cytotoxic activity to E♂G2 cells of sera from individual rats is shown on Fig. 2. Cytotoxic antibodies rise between the 7th and the 13th days, and reach a peak value (256–1024) at day 20. Thereafter a slight diminution in titer was observed, just before the second injection. The second peak of maximal titer (1024) was already obtained 6 days after the third or the fourth immunization. The fifth and sixth injections only maintain the maximal titer which slightly declines (down to 512) 23 days after the last immunization.

In a control experiment where the rats were only given one injection of 400×10^6 cells, a single antibody peak of comparable magnitude was obtained at day 20, followed by a slow decrease in titer and by day 48, sera from the above mentioned rats showed a cytotoxic activity about 10 times lesser than that of repeatedly inoculated rats.

DISCUSSION

Immunologic studies of the response of syngeneic rats against the Gross virus induced lymphoma (C58NT)D have been performed by many investigators. However few studies provide information about the optimal methods of immunization to produce the highly cytotoxic antisera required for passive immunotherapy trials. Antisera have been either raised following repeated intraperitoneal inoculations of (C58NT)D cells [1] or harvested from animals developing a slowly growing tumour after a single subcutaneous inoculation [5, 6].

In our experiments, the use of an immunization schedule consisting in repeated subcutaneous injections appears to frequently give antisera with high activity against E♂G2 cells. The best immunization schedule appears to consist, as in group VIII, of an initial injection of 400×10^6 viable (C58NT)D cells, followed 4 weeks later by 5 weekly injections of 200×10^6 cells. Such a schedule yields highly cytotoxic antisera (gmt = 1330, with individual titers ranging from 400 to 3200) within a rather short time.

These results were confirmed by the kinetic study of the immune response which also showed that the number of booster injections must not be too high and can even be reduced to 2 or 3. This was further verified: when 22 rats were immunized according to protocol VIII and blood drawn 7 days after the second and the fifth booster injections, the antisera obtained showed identical titers. So, near maximal titers may be achieved 2–3 weeks after a single inoculation, but highly cytotoxic antisera were only reliably obtained after repeated inoculations. Previously reported kinetic studies showed a biphasic response to a single inoculation of (C58NT)D cells in 8 week-old W/Fu rats: a first 19S and a second 7S antibody peaks were evidenced respectively 10 and 30–50 days after the subcutaneous injection of 100×10^6 cells [6]. In the experiments reported here, a single antibody peak was observed. An explanation for these appatently conflicting results could be that two different targets cells were used in the tests, which could have revealed different cell surface antigens; likely, we cannot exclude a difference due to the source of rats or lymphoma cells, various sublines of the (C58-NT)D considerably varying in their ability to generate immune responses [7].

Enzyme treated (C58NT)D cells were also used in immunization of W/Fu rats [9] and gave a higher cytotoxic antiserum than untreated cells without detectable qualitative modification of antigens on (C58NT)D cells. But a modification of the capacity of the animals to recognize each of the different cell surface antigens cannot be excluded, eventually related to the immunization schedule used. Finally, it must be stressed that the antisera obtained in the studies reported here were assessed for their activity towards Gross virus-associated cell surface antigens. Data from other investigator [3] and preliminary results from our laboratory indicate that, using E♂G2 lymphoma cells as target, a complement dependent cytotoxicity assay detects almost exclusively antibodies to GCSAa. However, these sera would certainly also react with other antigens of the Gross agent, but it is not known whether such reactivity would parallel their cytotoxic activity.

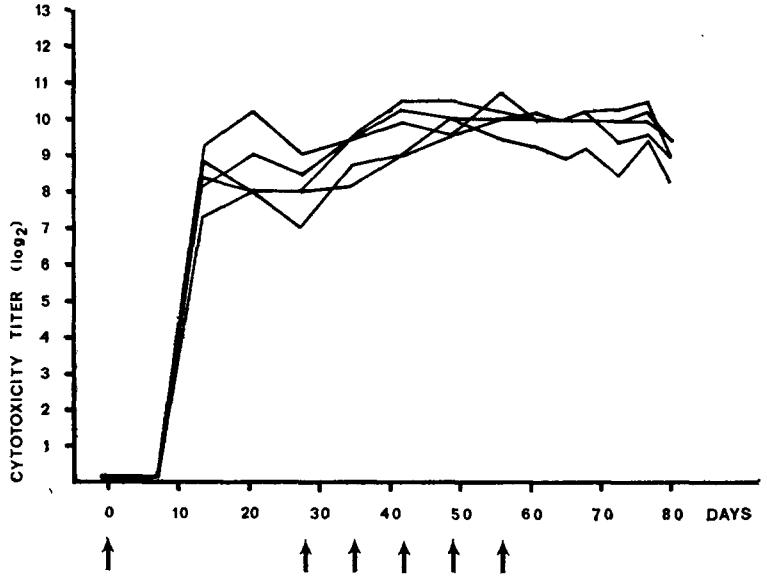

Fig. 2. Kinetics of cytotoxic activity of individual rat antisera during immunization according to schedule VIII. *Arrows indicate injection of lymphoma cells (see text).*

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The Difference Between "Selective Folinic Acid Protection" and "Folinic Acid Rescue" in L5178Y Cells Culture

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Abstract—*The separate concepts of "folinic acid protection" and "folinic acid rescue" have been investigated in two sublines of L5178Y lymphoblasts differing in their sensitivity to methotrexate (MTX). The effects on cell viability of both sublines have been studied using combinations of folinic acid (CF) with both MTX or DDMP (2, 4-diamino-5-3′, 4′-dichlorophenyl-6-methylpyrimidine).*

The use of CF protection (i.e. the simultaneous addition of CF) with MTX or DDMP produces different results in both cell lines. MTX-sensitive cells can be protected from the lethal effects of both MTX and DDMP by the simultaneous addition of CF. However, it is not possible to protect MTX-resistant cells in this way from the cytotoxic effect of either of these drugs. These results may partly be explained by transport studies which show that the simultaneous addition of CF has no effect on the efflux of both drugs from either cell line nor on MTX influx. Concurrent addition of CF does, however, reduce the influx of DDMP into MTX-sensitive cells but not into MTX-resistant cells.

Folinic acid rescue (i.e. the addition of CF some time after drug administration) rescues MTX-sensitive cells from the toxicity of MTX to a greater degree than MTX-resistant cells. However, rescue from DDMP toxicity occurs in both cell lines to a similar extent. The delayed addition of CF increases the efflux of MTX from MTX-sensitive cells, but not MTX-resistant cells. CF rescue does not alter DDMP efflux from either cell line.

These findings suggest that in treating neoplasms likely to be resistant to MTX selective folinic acid protection should be used, both with DDMP and/or MTX (i.e. CF should be given simultaneously with either or both drugs). This will prevent toxicity to cells likely to be sensitive to MTX (e.g. bone-marrow, gut) but will not prevent any tumouricidal effect of either agent on MTX-resistant cells. Conversely, in an MTX-sensitive tumour, the use of MTX plus a folinic acid rescue is more appropriate. Most of the therapeutic effect of DDMP will be lost by using CF rescue in treating either MTX-sensitive or MTX-resistant tumour cells.

INTRODUCTION

THE REVERSAL of methotrexate (MTX) toxicity by the addition of folinic acid (CF) is now a well established fact. This practice improves the therapeutic index of methotrexate and is widely used in the clinical management of various human malignancies including epidermoid carcinomas of the head and neck and acute leukaemia in children [1–7]. CF was first shown to prevent the toxic effects of MTX in mice [8–10] and then the concept of folinic acid rescue was demonstrated in mice bearing the L1210 leukaemia by Goldin *et al.* [11–13] and subsequently in man by Schoenbach *et al.* [14] and Burchenal and Kingsley-Pillers [15]. The logical extension of these earlier clinical and preclinical studies with folinic acid rescue enabled the use of very high doses of MTX over short periods of time

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(i.e. less than 36 hr) [16] and subsequently in lung cancer [17] and childhood tumours [18, 19].

The original experimental studies of Goldin *et al.* [12, 13] showed in particular that delayed administration of CF following treatment with MTX rescued from the toxic effects of the MTX without significantly reducing therapeutic effects, whilst simultaneous addition of CF and MTX prevented toxicity but resulted in significant loss of therapeutic activity. We have investigated the apparent differential effects of these two processes in tissue culture, namely "folinic acid rescue" involving subsequent addition of folinic acid after MTX, and "folinic acid protection" where the drug and the antidote were added simultaneously. From these studies it would be possible to determine whether particular advantages could be gained by exploiting either or both these concepts selectively.

We have shown *in vitro* that in an MTX-sensitive line of L5178Y lymphoblasts the simultaneous addition of equimolar amounts of MTX and CF significantly reduces the lethal effect of MTX [20]. However, in an MTX-resistant line CF failed to prevent the cytotoxic effects of MTX. This "selective protection" with CF of cells sensitive to MTX has been demonstrated also using DDMP, a diaminopyrimidine (i) in experimental transport studies [21] and (ii) clinically, where the simultaneous administration of CF with DDMP protects the bone marrow from the toxic effects of the drug without impairing the therapeutic effects against the tumour [22–25].

In this study we have compared the effects of (i) delayed and (ii) simultaneous addition of equimolar folinic acid with either MTX or DDMP on the colony-forming ability of two sublines of L5178Y lymphoblasts, one sensitive and the other resistant to MTX. In addition, the effects of CF on the extent of uptake and efflux of isotopically labelled MTX and DDMP by both cell lines was measured. Our results have enabled us to distinguish between the effects of "protection" and "rescue" with CF and illustrate that "protection" appears more selective for MTX-sensitive cells than "rescue". It may be particularly advantageous, therefore, to try to exploit this phenomenon when attempting to eradicate cells resistant to MTX from mixed cell populations.

MATERIAL AND METHODS

Chemicals and reagents

DDMP and DDMP-2-^{14}C were kindly provided for these studies as gifts from Drs. A. H. Griffith and C. A. Nicol, The Wellcome Research Laboratories, Burroughs Wellcome Co., Beckenham, Kent, U.K. and Research Triangle Park, N.C., U.S.A. The radiochemical purity of DDMP-2-^{14}C (specific activity 13.8 mCi/m mole) was 98%, as checked by TLC in three systems and by isotopic dilution. MTX and folinic acid (CF), as calcium leucovorin, were obtained from Lederle Products Limited, Montreal, Quebec, Canada and thymidine was obtained from Sigma Chemical Co., Norbiton, Surrey, U.K. ^{3}H-MTX (specific activity 250 mCi/m mole) was purchased from Amersham Searle Corporation, Illinois, U.S.A. and checked for radiochemical purity by paper chromatography in (a) 0.5% sodium carbonate solution and (b) *n*-butanol: pyridine:water (1:1:1). Other chemicals were purchased from Fisher Scientific Co. Ltd., Don Mills, Ontario, Canada, Sigma Chemical Co., or British Drug Houses, Limited.

Cell cultures

Two sublines of L5178Y lymphoblasts were grown in suspension culture in Fischer's medium for leukaemic cells in mice (Grand Island Biological Supplies Ltd., N.Y.) supplemented with 10% foetal calf serum (Flow Laboratories Ltd.) as described previously [26, 27]. The growth of the MTX-sensitive line was inhibited by continuous exposure to 10^{-8}M MTX and that of the MTX-resistant line by 5×10^{-6}M MTX. The MTX-resistant line was shown to transport MTX less effectively than the parent sensitive line, with results comparable to those published earlier [20, 28].

The cells, in logarithmic growth, were incubated for varying times with drugs at a concentration of 5×10^{-5}M. This drug concentration was selected since it produced a significant cell kill even in the drug-resistant cell line [27]. The viability assays were performed according to the method of Chu and Fischer [29] with the modifications described previously [26, 27]. Under these conditions the cloning efficiency was 60–75%. Treated cultures were expressed as a percentage of the control (non-treated) cultures, which were given the value of 100%. The mean and standard deviation of the colony counts of 5 replicate cultures were computed to attain the survival curves which were produced by plotting the percentage surviving fraction against time on a semi-logarithmic scale.

(i) "*Folinic acid protection*". This procedure involved the simultaneous addition of equimolar CF with the drug to the cultures at the

commencement of the experiment, as described previously [20].

(ii) "*Folinic acid rescue*". This procedure involved the addition of drug (MTX or DDMP) at zero time followed by the subsequent addition of equimolar CF to individual cultures at 6, 24 and 48 hr after the experiment commenced. Prior to this addition of CF, the cells were removed from the drug-containing medium by centrifugation at 500 *g* and resuspended in fresh medium. At 24 hr after CF addition the viability assays were carried out, namely at 30, 48 or 72 hr after the original addition of MTX or DDMP. This procedure was an attempt to duplicate the *in vivo* conditions of rescue with CF.

(iii) *MTX reversal by thymidine.* This procedure involved the simultaneous addition of 4×10^{-6}M thymidine with the drug to the cultures at the commencement of the experiment.

Transport studies

Logarithmically-growing L5178Y cells were removed from suspension by centrifugation at 350 *g* at room temperature for 3 min. The cells were then counted and resuspended to a final concentration of 2×10^6/ml in Hank's balanced salt solution pH 7.4 (Grand Island Biologicals Ltd., N.Y.) and placed in a water bath at 37°C, as described previously [20, 21, 30]. Drugs were added to aliquots of these cell suspensions resulting in a final drug concentration of 5×10^{-5}M and a radio-isotope concentration of 0.625 μCi/ml and 2.5 μCi/ml of ^{14}C-DDMP and ^{3}H-MTX respectively. Incubations were carried out at 37°C in a water-bath with gentle shaking.

(i) *Influx.* At various times after drug addition (either drug alone or drug plus equimolar CF), aliquots of cell suspension were removed, washed with ice-cold Hank's solution and isotonic saline as described before [20, 21, 30]. The resultant cell pellets were solubilised in N. NaOH and mixed with 10 ml of Aquasol (NEN, Dorval, Quebec, Canada). Radioactivity was determined using a Nuclear Chicago Scintillation Counter Unilex II Model No. 6853 at 63% efficiency for ^{14}C and 40% efficiency for ^{3}H.

For long term incubations with DDMP alone or in combination with equimolar CF the drugs were added to cells growing in Fischer's medium supplemented with 10% foetal calf serum. Six hours later the incubation was terminated by rapid chilling and dilution with ice-cold Hank's solution as described above, and the ^{14}C content of the resultant cell pellets was estimated.

(ii) *Efflux.* Previous experiments had established that after a 30 min incubation a steady-state concentration of both DDMP and MTX was achieved in both cell lines [20, 21, 30]. Therefore, the rates of efflux of DDMP or MTX from the cells at 37°C were determined by incubating cells with the drug, using the conditions described above, for 30 min. The cells were then centrifuged at 350 *g* for 2 min at room temperature and resuspended to the same volume in fresh Hank's solution without drug at 37°C. In these experiments the rate of efflux of the drugs alone, previously established [20, 21], was confirmed and the effects of (a) the simultaneous addition of CF with the drugs at the commencement of the preincubation, or (b) the delayed addition of CF until the cells were resuspended in fresh drug-free medium after the initial 30 min incubation, on the rates and extents of drug efflux were determined. Aliquots of cell suspension were removed at subsequent time intervals, and treated as described above in order to determine their radioactivity content.

RESULTS

Cell viability assays demonstrating "folinic acid protection"

Figure 1 shows the effect of 5×10^{-5}M MTX in the presence and absence of simultaneously added equimolar folinic acid on the survival of MTX-sensitive (Fig. 1a) and MTX-resistant (Fig. 1b) L5178Y cells. These results confirm our earlier findings (i) that the simultaneous addition of folinic acid with the MTX had no effect on the response of resistant L5178Y cells to MTX, however (ii) the lethal effects of MTX on the sensitive cells were considerably reduced by exposure to the combination with only a 0.75 log kill after 48 hr [20].

This concept of selective folinic acid protection of MTX-sensitive cells is also illustrated by the results in Fig. 2. MTX-sensitive L5178Y cells were treated with 5×10^{-5}M DDMP alone or with the simultaneous addition of equimolar folinic acid (Fig. 2a). The combination almost abolished the reduction in cell viability seen with DDMP alone. In contrast in the MTX-resistant cells (Fig. 2b) no protection was afforded by CF.

These data indicate that there are significant differences in sensitivity to folinic acid protection in two sublines of L5178Y lymphoblasts. It is not possible to protect MTX-resistant cells from the toxicity of either MTX or DDMP by the simultaneous addition of

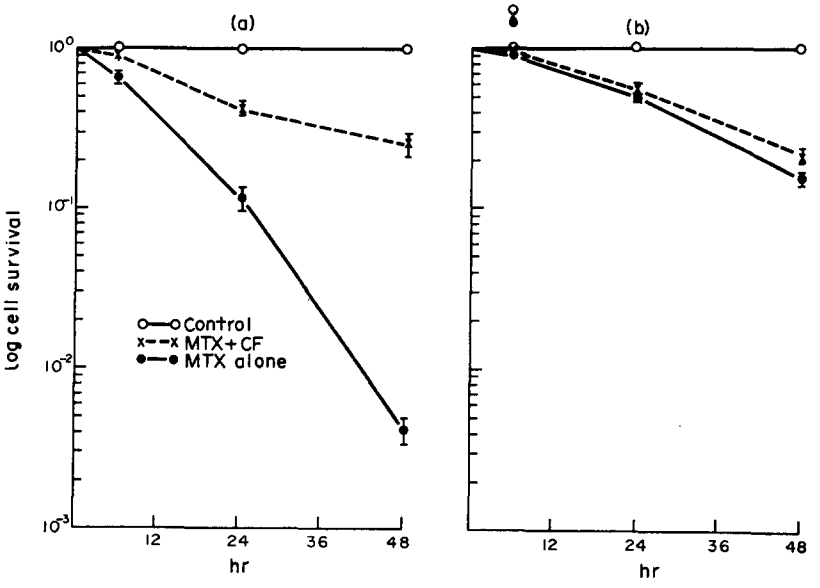

Fig. 1. The ability of CF to "protect" from MTX cytotoxicity. The effects of a 6-, 24- and 48-hr exposure to 5×10^{-5}M MTX alone or in combination with the simultaneous addition of 5×10^{-5}M CF on the colony-forming ability of L5178Y cells. (a) MTX-sensitive cells; (b) MTX-resistant cells. Percentage surviving fraction plotted against duration of drug exposure. Each point represents the mean of 5 determinations.

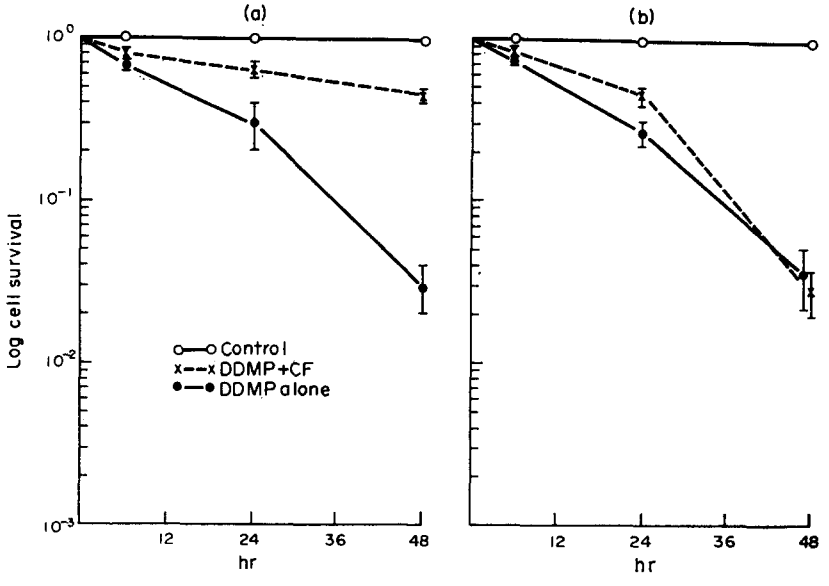

Fig. 2. The ability of CF to "protect" from DDMP cytotoxicity. The effects of a 6-, 24- and 48-hr exposure to 5×10^{-5}M DDMP alone or in combination with the simultaneous addition of 5×10^{-5}M CF on the colony-forming ability of L5178Y cells. (a) MTX-sensitive cells; (b) MTX-resistant cells. Percentage surviving fraction plotted against duration of drug exposure. Each point represents the mean of 5 determinations.

equimolar CF. However, this inability to protect MTX-resistant cells should not be taken to imply that drug toxicity cannot be overcome in these cells, since Fig. 3 shows that the simultaneous addition of equimolar thymidine and MTX reverses the lethal effects of MTX not only in MTX-sensitive cells but also in the drug resistant cells.

Cell viability assays demonstrating "folinic acid rescue"

Figures 4 and 5 illustrate CF rescue from the lethal effects of MTX and DDMP respectively, using drug concentrations of 5×10^{-5}M. In these experiments, as described in Material and Methods folinic acid was administered 6, 24 and 48 hr after drug addition at zero time,

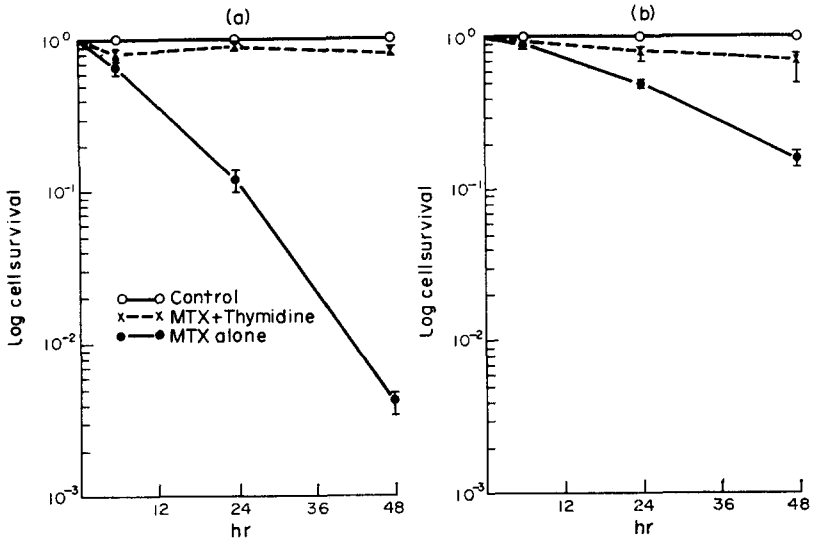

Fig. 3. The ability of thymidine to reverse or protect from MTX cytotoxicity. The effects of a 6-, 24- *and* 48-hr *exposure to* 5×10^{-5}M *MTX alone or in combination with the simultaneous addition of* 4×10^{-6}M *thymidine on the colony-forming ability of L5178Y cells. (a) MTX-sensitive cells; (b) MTX-resistant cells. Percentage of surviving fraction plotted against duration of drug exposure. Each point represents the mean of* 5 *determinations.*

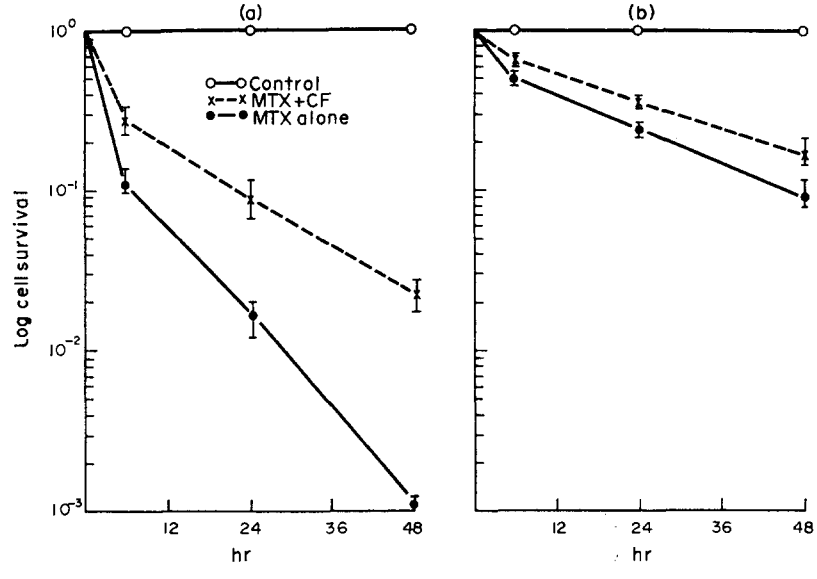

Fig. 4. The ability of CF to "rescue" from MTX induced cytotoxicity. The effects of exposure to 5×10^{-5}M *MTX alone or in combination with subsequent addition of equimolar CF after* 6-, 24- *and* 48-hr *exposure to MTX on the colony-forming ability of L5178Y cells. Viability assays were carried out* 24 hr *after the addition of CF to the cells in the drug containing medium, namely* 30-, 48- *and* 72-hr *after the experiment commenced. (a) MTX-sensitive cells; (b) MTX-resistant cells. Percentage surviving fraction plotted against the time when the CF was added after the commencement of the experiment. Each point represents the mean of* 5 *determinations.*

and the ability of cells to form colonies was tested 24 hr later after the addition of CF, i.e. at 30, 48 and 72 hr after the start of the experiment. Figure 4 (a) shows that MTX-sensitive cells can be rescued from the lethal effects of MTX, and Fig. 4 (b) shows that the subsequent addition of CF after MTX treatment of MTX-resistant cells does afford a slight rescue. The extent of rescue after 6 hr treatment with MTX is comparable in both sensitive and resistant cells (i.e., approx. 40%), but after 24 and 48 hr treatment the addition of CF rescues the sensitive cells more effectively than the drug resistant line (see Table 1).

The results with DDMP in Fig. 5 also show

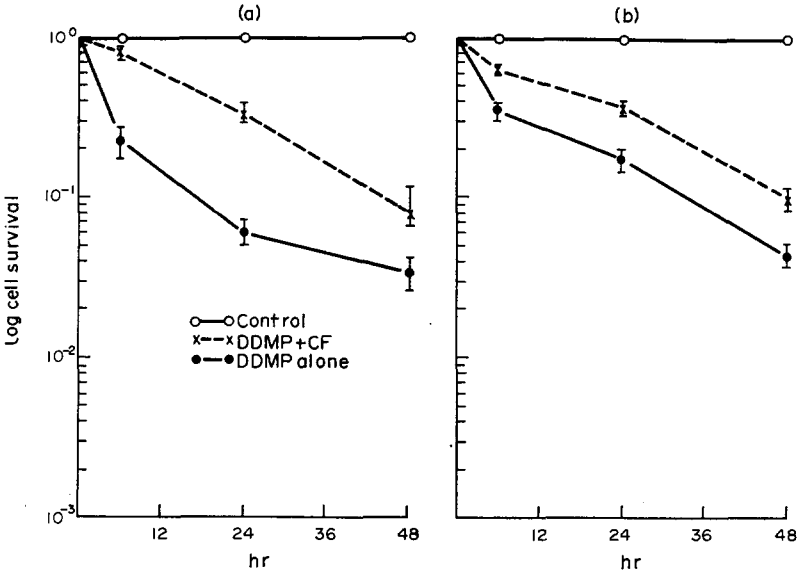

Fig. 5. The ability of CF to "rescue" from DDMP induced cytotoxicity. The effects of exposure to 5×10^{-5}M *DDMP alone or in combination with subsequent addition of equimolar CF after* 6-, 24- *and* 48-hr *exposure to DDMP on the colony-forming ability of L5178Y cells. Viability assays were carried out* 24 hr *after the addition of CF to the cells in the drug-containing medium, namely* 30-, 48- *and* 72-hr *after the experiment commenced. (a) MTX-sensitive cells; (b) MTX-resistant cells. Percentage surviving fraction plotted against the time when the CF was added after the commencement of the experiment. Each point represents the mean of* 5 *determinations.*

*Table 1. The differential effects on cell survival of "protection" and "rescue" with CF in combination with MTX or DDMP treatment**

	Percentage of cell survival* MTX-sensitive cells	MTX-resistant cells
MTX treatment		
With CF—rescue	44%	26%
With CF—protection	75%	11%
With added thymidine	96%	83%
DDMP treatment		
With CF—rescue	25%	26%
With CF—protection	78%	0%

*These numbers were calculated from the results illustrated graphically in Figs. 1–5, using the 48 hr points in all cases. They represent the percent increased survival after addition of CF or thymidine compared with survival from drug treatment alone.

that it is possible to rescue both cell lines to a degree from DDMP toxicity by the delayed addition of folinic acid. A better rescue is achieved in the sensitive cells when the rescue is commenced within 24 hr of DDMP treatment with 85% and 60% of the population being rescued when CF was added at 6 and 24 hr respectively. When rescue is delayed to 48 hr the extent of rescue is much less and both MTX-sensitive and MTX-resistant cells appear rescued to a similar degree (see Table 1).

These data indicate that in applying the principle of folinic acid rescue both cell types can be rescued to a certain extent from the toxicity of both DDMP and MTX. The sensitive cells are rescued to a greater degree than the resistant cells from both drugs unless the DDMP rescue is delayed until 48 hr when this differential is lost.

Transport studies

(i) *Influx of MTX and DDMP.* We have previously shown, in both cell lines, that the simultaneous addition of equimolar folinic acid is without effect on the steady-state level of MTX after a 30 min *in vitro* incubation [20]. Similarly, the uptake of DDMP into MTX-resistant cells was unaffected by the presence of CF [21]. However, during this short-term incubation the extent of influx of DDMP into the MTX-sensitive cells was markedly reduced by the simultaneous addition of folinic acid [21, 30]. In these present studies we have confirmed these latter observations after a 6 hr incubation with 5×10^{-5}M DDMP in the presence and absence of simultaneously added equimolar CF. The results in Table 2 demonstrate that the simultaneous addition of CF, under the conditions used for the cell viability assays described above, selectively reduces the

*Table 2. The effect of simultaneous addition of folinic acid on the level of DDMP in L5178Y lymphoblasts after a 6-hr incubation at 37°C**

Cell line	Extent of DDMP uptake†
MTX-sensitive	49% ± 5
MTX-resistant	88% ± 3

*Uptake of ^{14}C-DDMP was measured following a 6 hr exposure of logarithmically growing cells in standard culture conditions at a concentration of approximately 2×10^5 cells per ml (as described in Material and Methods) to 5×10^{-5}M DDMP, plus or minus equimolar folinic acid.

†The extent of uptake in the absence of added folinic acid = 100%.

amount of DDMP which enters MTX-sensitive cells without markedly influencing the uptake of the drug by the MTX-resistant cells.

These results are in agreement with our previous proposal that the inability of folinic acid to prevent DDMP influx into MTX-resistant cells may account possibly for the failure of folinic acid to protect this cell line from DDMP toxicity [21].

(ii) *Efflux of MTX and DDMP.* L5178Y lymphoblasts were preloaded with either MTX or DDMP by incubation for 30 min at 37°C with drug at a concentration of 5×10^{-5}M. In both cases more drug is taken up by MTX-sensitive cells than by the resistant cells, confirming previously reported observations [20, 21, 28]. The cells were then centrifuged, washed and resuspended either in fresh medium alone or medium containing 5×10^{-5}M CF at 37°C. Aliquots of cell suspension were removed at the time shown in Figs. 6 and 7 for MTX and DDMP respectively. The Figs. illustrate the extent of efflux of the drugs in the presence and absence of CF from the two cell types. In Fig. 6 it can be seen that the addition of CF markedly enhances both the initial rate and final extent of efflux of MTX from the MTX-sensitive cells. This is in contrast to the lack of effect that added CF has on drug efflux from MTX-resistant cells. Figure 7 shows that the addition of CF to cells preloaded with DDMP does not alter the efflux characteristics from either cell type. The addition of folinic acid prior to preloading the cells with either drug was without effect on the subsequent efflux of either DDMP or MTX in both cell lines of L5178Y cells [unpublished data, 20].

These results indicate that the original addition of folinic acid enhances the efflux from MTX-sensitive cells, but is without effect on MTX-resistant cells or on DDMP efflux from both cell types.

DISCUSSION

These studies were designed to answer the following questions about the reversal or prevention of MTX or DDMP toxicity:

(i) Is there a difference between the MTX-

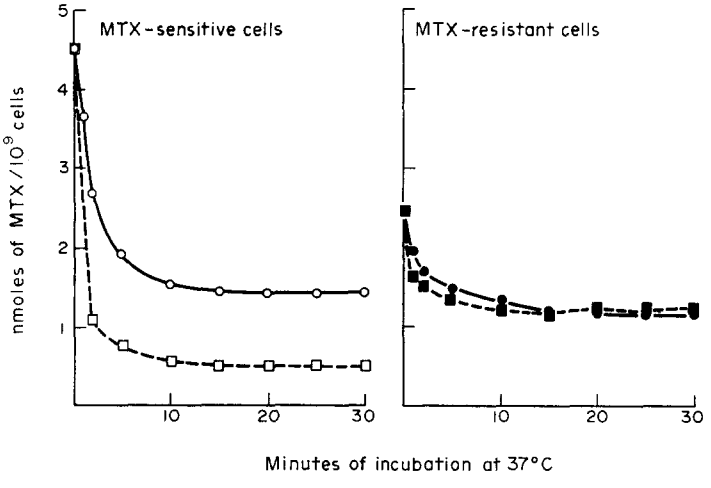

Fig. 6. The rate of efflux of ^{3}H-MTX from "preloaded" MTX-sensitive and MTX-resistant L5178Y cells expressed in n moles per 10^9 cells as a function of time of re-incubation either in drug-free medium alone or in drug-free medium plus 5×10^{-5}M CF. Each point represents the mean of 6 determinations. Overall scatter not in excess of 10%.

MTX-sensitive cells : *MTX alone* ○——○
: *MTX+CF* □- - -□
MTX-resistant cells : *MTX* ●——●
: *MTX+CF* ■- - -■

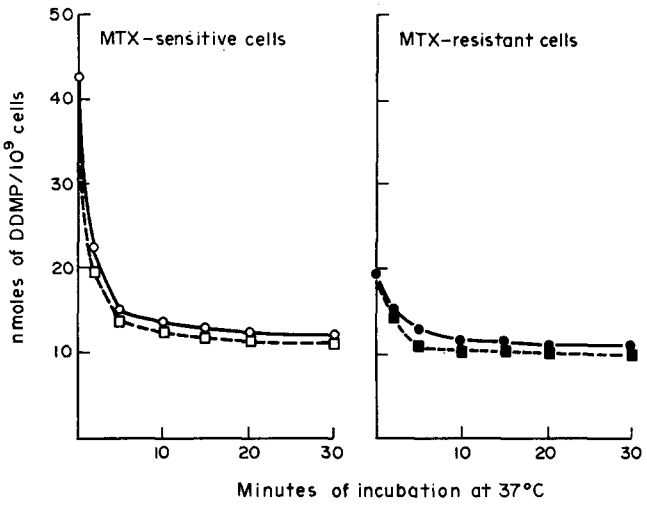

Fig. 7. The rates of efflux of ^{14}C-*DDMP from "preloaded" MTX-sensitive and MTX-resistant L5178Y cells expressed in n moles per* 10^9 *cells as a function of time of reincubation either in drug-free medium alone or in drug-free medium plus* 5×10^{-5}M *CF. Each point represents the mean of* 6 *determinations. Overall scatter not in excess of* 10%.

MTX-sensitive cells : *DDMP alone* ○——○
: *DDMP+CF* □- - -□
MTX-resistant cells: : *DDMP alone* ●——●
: *DDMP+CF* ■- - -■

sensitive and MTX-resistant cell lines in their response to MTX or DDMP using CF protection as opposed to CF rescue?
(ii) Is there any correlation between the biological viability assays and the transport phenomena seen when studying DDMP or MTX alone or in combination with CF?
(iii) If a distinction can be made between CF "rescue" and "protection" can it provide a basis for separate therapeutic strategies against varied tumour cell types?
(iv) From our experimental data, can we provide any insight into the clinical observations that have been made with DDMP?

Our data show that the effects on cell viability if CF is added simultaneously with MTX or DDMP (namely "CF protection") are very different from those observed following the delayed addition of CF (namely "CF rescue"). A summary of results is given in Table 1.

When treating cells with MTX a significantly greater proportion of MTX-sensitive cells can be protected for a given cell kill than can be rescued following delayed addition of CF. This protection of MTX-sensitive cells confirms our earlier results with MTX and pyrimethamine [20], those of Burchenal *et al.* with DDMP [31] and the work of Borsa and Whitmore [32]. In MTX-resistant cells, however, simultaneous addition of CF affords no protection whilst the delayed addition does rescue a small proportion of the cell population. An explanation for this latter finding may be that under conditions of rescue, the extracellular MTX is washed from the cells, preventing further cell kill, whilst for protection the MTX remains in contact with the cells for the duration of the experiment. The greater degree of protection afforded to MTX-sensitive cells with simultaneous CF may be related to the fact that the CF is either competing with, or attenuating the MTX toxicity throughout the experiment [21, 30]. The observed rescue of MTX-sensitive cells, confirming the now well documented phenomenon of leucovorin rescue [33–35], may be explained by the observation that delayed CF addition enhances the efflux of MTX. This latter finding was one of several mechanisms which have been implicated in the CF reversal of MTX toxicity [20, 33]. However, this efflux effect is not seen in the MTX-resistant cells and neither are they rescued to the same extent.

The effects of simultaneous addition of equimolar CF to DDMP treated cells are similar to those with MTX, with a higher degree of protection afforded to MTX- sensitive cells and negligible protection in MTX-resistant cells. In these sensitive cells, the simultaneous addition of CF must, as in the case of MTX, be reducing the toxicity of the

DDMP. In contrast, with the delayed addition of CF there is less selectivity and if rescue is delayed until after 24 hr both drug-sensitive and -resistant cells are rescued to approximately the same extent (see Table 1). The selective protection afforded to MTX-sensitive cells by simultaneous addition of CF may occur as a result of the competition for uptake seen between DDMP and CF in this cell line [21, 30]. This competition is not apparent in drug-resistant cells which are also not protected from the cytotoxicity of DDMP by simultaneous CF addition. In contrast the delayed addition of CF allows some degree of rescue in both cell lines. This effect cannot be explained in terms of altered efflux of DDMP. The observation that the extent of rescue with equimolar CF is not as great from DDMP as it is from MTX in MTX-sensitive cells, may be related to the fact that in this experimental system DDMP has a relatively poor ability to inhibit dihydrofolate reductase as compared with MTX [27].

In treating with MTX clinical neoplasms which are primarily composed of cells which are relatively resistant to MTX by virtue of similar mechanisms to those in these L5178Y cells, the simultaneous MTX-CF combinations would be expected to produce less cytotoxic effects against normal cell systems, i.e. bone marrow and gut, without loss of antitumour effect. In addition, MTX treatment could be given for longer than usual. Conversely, a clinical tumour that has properties akin to the sensitive-L5178Y cells and normal sensitive cell systems would be largely protected from MTX toxicity by simultaneous CF. In such tumours, delayed CF or CF rescue would be the appropriate therapy. Since the use of thymidine in combination with MTX reverses the toxicity in both MTX-sensitive and MTX-resistant cells to comparable extents (see Fig. 3 and Table 1), there would appear to be no selectivity and therefore no advantage in employing this combination.

Most of the therapeutic effect of DDMP will be lost if the drug is combined with the delayed addition of CF or CF rescue in either MTX-sensitive or -resistant cells. DDMP would be expected to be most effective against resistant-type cells in which CF protection (simultaneous addition of CF) is employed. This correlates well with our clinical experiences [22–25] and the original clinical observations of Murphy *et al.* [36].

CONCLUSION

The optimal MTX-CF combinations will be determined by whether the tumour being treated has properties more closely related to the MTX-resistant type or MTX-sensitive type cells. In the former case, CF protection strategy would be expected to be the most effective and in the latter case, the usual delayed rescue would be the most rational.

The optimal combination of DDMP and CF against MTX-resistant cells is the simultaneous addition of the two drugs. In this way, selective folinic acid protection of drug sensitive cells can be exploited. However, this selectivity is lost when DDMP is administered followed by a CF rescue.

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Studies on the Toxicity and Antitumour Activity of Prednimustine, a Prednisolone Ester of Chlorambucil*

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Abstract—*Preliminary results are reported on the acute toxicity and antitumour effectiveness of prednimustine, a prednisolone ester of chlorambucil. Prednimustine was less toxic to the rat, but slightly more toxic to the mouse than chlorambucil. In both rodent species, the acute toxicity of binary combinations of chlorambucil and prednisolone could be reduced by giving the steroid 4 hr after the alkylating agent.*

Prednimustine was more effective than chlorambucil against a sensitive line of the Yoshida ascites sarcoma and killed 70% of cells from a strain which exhibited a 50-fold acquired resistance to chlorambucil. However, prednimustine was ineffective against the Ehrlich ascites tumour which is intrinsically resistant to alkylating agents.

The molecular mode of action of prednimustine appeared comparable to that of chlorambucil, though the former compound did exhibit corticosteroid-like properties, as evidenced by its cytotoxicity to a hormone-sensitive mouse lymphoma cell line.

INTRODUCTION

SINCE the initial recognition of the leukopoenic properties of nitrogen mustard and its utilisation for the treatment of leukaemia [1], many alkylating compounds have been synthesised in attempts to produce selective antitumour agents. Alkylating functions have been attached to biologically essential molecules, for example sugars or amino acids, in the hope that the resultant compounds might be transported selectively into proliferating cells [2, 3]. Such a rationale has provided many of the alkylating agents which currently find wide clinical application (e.g. myleran, melphalan, degranol, chlorambucil) [2–5]. More recently alkylating functions have been attached to various hormones by workers at the Leo Research Laboratories, Sweden [6]. One of these compounds, Estracyt, a 3-*N*-(bis(2-chloroethyl) carbamate of estradiol 17-phosphate, is useful in the treatment of prostatic carcinoma [7–9].

The present communication describes the results of animal and tissue culture studies with another steroid–cytostatic compound, prednimustine, a prednisolone-21 ester of chlorambucil [10, 11].

MATERIAL AND METHODS

Chemicals

Prednimustine (pregna-1, 4-diene-3, 20-dione, 11, 17-dihydroxy, 21-4-p(bis-(2-chloroethyl)-amino) phenylbutyrate was synthesised in the laboratories of Leo Research, Helsingborg, Sweden. Chlorambucil (Leukeran) ($ClCH_2$-$CH_2)_2N.\ _6H_4.\ (CH_2)_3COOH$ was synthesised in the Chester Beatty Research Institute, London and prednisolone (11β, 17α, 21-trihydroxy-1, 4-pregnadiene- 3, 20-dione) was obtained from the Sigma Chemical Company Ltd., Kingston-upon-Thames, England. It should be noted that the molecular weights of prednimustine, chlorambucil and prednisolone are respectively 646.7, 304.2 and 360.5: for

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rough comparative purposes, therefore, xg prednimustine contains $x/2$ g chlorambucil. Fischer's medium, foetal bovine serum and horse serum were obtained from Gibco-Biocult, Glasgow, Scotland. Benzyl penicillin (Na) BP and streptomycin sulphate BP were obtained from Glaxo Laboratories Ltd., Greenford, England. [6-^{3}H]-thymidine (25Ci/mmol), [6-^{3}H]-uridine (5Ci/mmol), L-[4, 5-^{3}H]-leucine (1Ci/mmol) and 2-deoxy-D-[1-^{3}H]-glucose (4.6Ci/mmol) were all obtained from the Radiochemical Center, Amersham, England All other chemicals were purchased from either Hopkin and Williams Ltd., Chadwell Heath, Essex, England or British Drug Houses Ltd., Poole, Dorset, England. AnalaR grades were used where available.

Cell lines

Two lines of the Yoshida ascites sarcoma, one sensitive to alkylating agents and the other exhibiting a 50-fold [12] increase in acquired resistance, were routinely passaged at weekly intervals by the intraperitoneal injection of 2×10^6 cells in female Wistar rats as previously described [13]. Ehrlich ascites tumour cells were also passaged weekly by an intraperitoneal injection of 2×10^6 cells in male BALB/C mice. Drugs were administered subcutaneously three days after tumour transplantation, dissolved in dimethyl sulphoxide (DMSO). Rats or mice were killed by cervical dislocation and the tumour aspirated in 0.9% saline.

Primary suspension cultures of both the sensitive and resistant Yoshida sarcoma cell lines could be maintained for up to 10 days *in vitro*. Growth was initiated with 5×10^4 cells/ml in Fischer's medium containing 20% foetal bovine serum, benzyl penicillin (60μg/ml) and streptomycin (100μg/ml). Drugs were added to suspension cultures 24 hr after initiation when the cells were in logarithmic growth.

Hormone-sensitive mouse lymphoma cells (S49) were maintained in logarithmic growth in suspension culture using Fischer's medium supplemented with 10% horse serum. Drugs were added 24 hr after initiation of new cultures as described for Yoshida cells.

Survival curves

The survival of female Wistar rats and male BALB/C mice from acute drug treatments was determined: all animals were weighed daily for at least 3 weeks. Drugs were administered subcutaneously.

Yoshida and Ehrlich ascites tumour cells were harvested from the peritoneal cavity of both drug-treated and control animals and counted using a Coulter Model ZF Electronic Particle Counter (Coulter Electronics, Harpenden, Herts, England). The surviving fraction was determined from the percentage of cells surviving in the treated animals compared with those present in untreated hosts.

Survival data for the S49 mouse lymphoma cells were determined by growth rate and trypan blue dye exclusion tests. Drugs were added to *in vitro* cultures in either ethanol or DMSO; the final concentrations of ethanol or DMSO were never more than 0.1%.

Isotope incorporation experiments

Yoshida ascites sarcoma cells were harvested from animals treated on the 4th day following tumour transplantation with either a single dose of chlorambucil (10 mg/kg), or prednisolone (10 mg/kg), or prednimustine (20 mg/kg). At various times after treatment animals were killed and aliquots of cells(10^6/ml) were incubated in Fischer's medium in the presence of ^{3}H-thymidine (1μCi/ml), ^{3}H-uridine (5μCi/ml) or ^{3}H-leucine (5μCi/ml) for 15 min at 37°C. At the end of the incubation the cell suspensions were cooled to 0°, centrifuged to remove medium and extracted twice with an equal volume of 0.2 N perchloric acid (PCA). The residual pellet was fractionated into RNA, DNA and protein by the method of Munro and Fleck [14]. The radioactivity in aliquots of the DNA, RNA and protein fractions was counted using a Multimat II Intertechnique liquid scintillation spectrometer. DNA was estimated colorimetrically by the method of Burton [15], RNA by the Orcinol technique [16] and protein according to Lowry *et al.* [17]. The packed cell volume of a constant number of cells at various times after treatment was determined using haematocrit tubes.

Measurement of glucose transport in Yoshida sarcoma cells

Tumour-bearing animals received prednimustine (20 mg/kg) or prednisolone (10 mg/kg). The tumour cells were harvested 3 hr later and incubated in Fischer's medium containing 1.0μCi/ml ^{3}H-2-deoxyglucose at 37°C. At various time intervals thereafter, aliquots of the cell suspension were cooled to 0°, centrifuged, washed with ice-cold Fischer's medium, and extracted with an equal volume 0.5 M perchloric acid. The radioactivity in aliquots of the supernatant was counted in a Multimat II Intertechnique liquid scintillation spectrometer.

Studies were also performed on Yoshida cells exposed *in vitro* to prednimustine (10^{-5}M) or prednisolone (10^{-5}M). ^{3}H-2-Deoxyglucose at a concentration of 0.1μCi/ml was added im-

mediately after the drug and the cells were incubated at 37°C. Aliquots of cell suspension were removed at measured time intervals, extracted with 0.5 N PCA and counted as above.

RESULTS

Acute toxicity of prednimustine

Prednimustine was less toxic to rats than chlorambucil, as shown in Table 1. For example, a dose of 32 mg/kg chlorambucil killed 3/10, whereas an equivalent dose of prednimustine (in terms of its chlorambucil content, 64 mg/kg) produced no deaths. The simultaneous administration of chlorambucil (32 mg/kg) and prednisolone (32 mg/kg) produced toxicity comparable to chlorambucil alone (32 mg/kg). However, when prednisolone (32 mg/kg) was given 4 hr after chlorambucil (32 mg/kg) the resultant toxicity was less than that produced either by chlorambucil alone (32 mg/kg) or by the simultaneous administration of chlorambucil (32 mg/kg) and prednisolone (32 mg/kg). Figure 1 shows selected body weight curves of rats following treatment with prednimustine, chlorambucil, or binary combinations of chlorambucil and prednisolone. Although the weight losses induced by high doses of prednimustine were less than those induced by chlorambucil, recovery was slower. At low drug doses there was little difference in the weight losses produced either by chlorambucil alone, or by prednimustine, or by combinations of the two agents.

BALB/C mice tolerated larger doses of both chlorambucil and prednimustine than did Wistar rats. However, as can be seen from Table 2, prednimustine (at doses equivalent to its chlorambucil content) was more toxic to the mouse than chlorambucil. Further, prednisolone (64 mg/kg) enhanced the toxicity of chlorambucil (64 mg/kg) when the two drugs were administered simultaneously, though when prednisolone (64 mg/kg) was given 4 hr after chlorambucil (64 mg/kg) the toxicity became comparable to that produced by chlorambucil (64 mg/kg) alone. From Fig. 2 it can be seen that prednimustine produced a larger fall in mouse body weight than did an equivalent dose of chlorambucil. The reduction in body weight produced by chlorambucil was enhanced by the simultaneous administration of prednisolone, and in addition recovery was delayed. The reduction in body weight resulting from binary combinations of chlorambucil and prednisolone was less extensive when the administration of the hormone was delayed by 4 hr.

Table 1. *Toxicity of prednimustine, chlorambucil and binary combinations of chlorambucil and prednisolone in Wistar rats*

Drug	Dose mg/kg †	Day of death *	21 day survivors
Chlorambucil	2	0	10
	4	0	10
	8	0	10
	16	0	10
	32	2^2, 3^1	7
	64	2^8, 8^2	0
Prednimustine	4	0	10
	8	0	10
	16	0	10
	32	0	10
	64	0	10
	128	14^1, 15^2	7
Chlorambucil + prednisolone (simultaneous)	2	0	10
	4	0	10
	8	0	10
	16	0	10
	32	10^1, 17^1, 18^1, 19^1	6
	64	1^8, 2^1, 20^1	0
Chlorambucil followed 4hr later by the same dose of prednisolone	32	0	10

* All drugs were injected subcutaneously.

† Superscripts indicate numbers of animals dying on day shown. Each group contained 10 animals

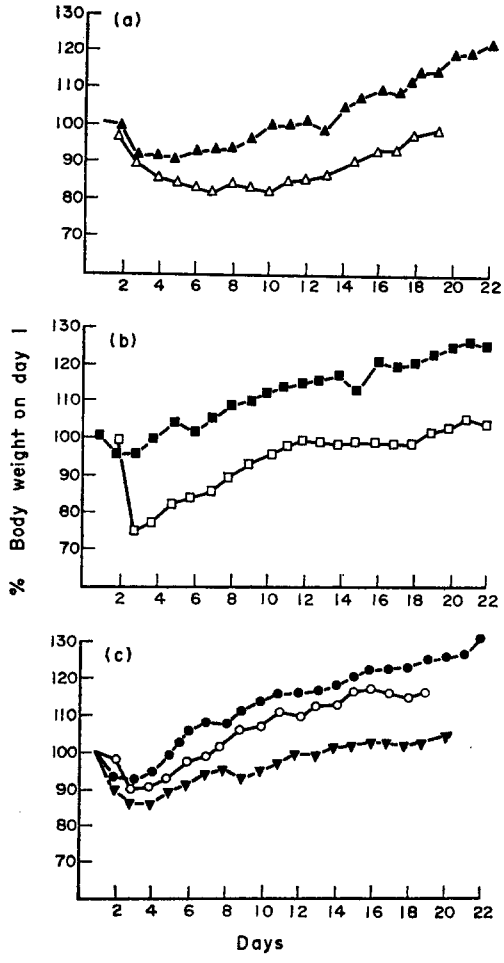

Fig. 1. Effect of prednimustine, chlorambucil or chlorambucil plus prednisolone on the body weights of female Wistar rats.

(*a*) ▲——▲ *prednimustine* 32 mg/kg;
△——△ *prednimustine* 64 mg/kg;
(*b*) ■——■ *chlorambucil* 16 mg/kg;
□——□ *chlorambucil* 32 mg/kg;
(*c*) ●——● *chlorambucil* 16 mg/kg *plus prednisolone* 16 mg/kg, *simultaneous*;
▼——▼ *chlorambucil* 32 mg/kg *plus prednisolone* 32 mg/kg, *simultaneous*;
○——○ *chlorambucil* 32 mg/kg *followed* 4 hr *later by prednisolone* 32 mg/kg.

All drugs were injected subcutaneously on day one. Ten animals were used to determine each point, with the exception of chlorambucil (32 mg/kg) *where* 2 *deaths occured on day* 2 *and* 1 *on day* 3. *Average scatter about each point* ⩾ ± 10%.

schedule of administration was critical. Simultaneous administration of chlorambucil and prednisolone produced the same survival of both tumour cell types as resulted from the injection of chlorambucil alone. However, by exploiting the observation that sub-cellular binding of chlorambucil was maximal 4 hr after injection [18, 19], and giving prednisolone at this time, the surviving fractions corresponded

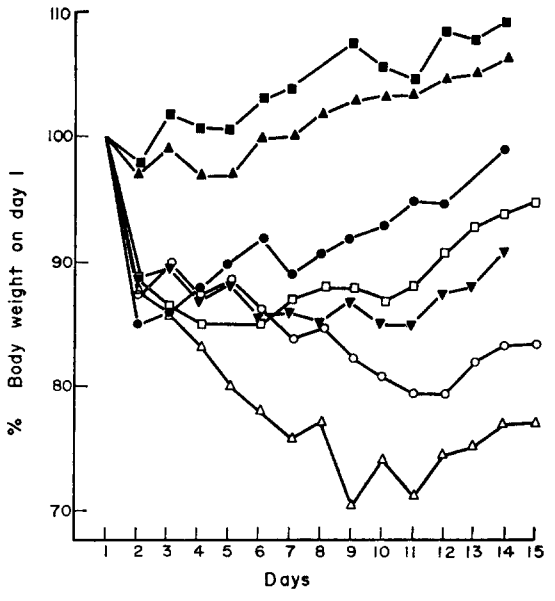

Fig. 2. Effect of prednimustine, chlorambucil or chlorambucil plus prednisolone on the body weights of BalbC mice.

▲——▲ *prednimustine* 16 mg/kg;
△——△ *prednimustine* 128 mg/kg;
■——■ *chlorambucil* 8 mg/kg;
□——□ *chlorambucil* 64 mg/kg;
●——● *chlorambucil* 8 mg/kg *plus prednisolone* 8 mg/kg *simultaneous*;
○——○ *chlorambucil* 64 mg/kg *plus prednisolone* 64 mg/kg, *simultaneous*;
▼——▼ *chlorambucil* 64 mg/kg *followed* 4 hr *later by prednisolone* 64 mg/kg.

All drugs were injected subcutaneously on day one. Ten animals were used to determine each point, with the exception of: prednimustine 128 mg/kg *where* 2 *deaths occurred on day* 8 *and* 1 *on day* 9; 64 mg/kg *chlorambucil followed by* 64 mg/kg *prednisolone, where* 5 *deaths occurred on day* 2. *Average scatter about each point* ⩾ ± 8%.

Antitumour effects of prednimustine

Dose–response curves for chlorambucil and prednimustine against alkylating agent-sensitive and resistant lines of the Yoshida ascites sarcoma in host rats are shown in Fig. 3. Prednimustine was more effective than chlorambucil against the sensitive tumour and killed a small but significant fraction of drug-resistant cells. Both tumour lines showed some sensitivity to prednisolone. The antitumour effects of prednimustine could be mimicked with combinations of prednisolone and chlorambucil, though the to those obtained with an equivalent dose of prednimustine. Thus at a dose of 10 mg/kg chlorambucil, followed 4 hr later by 10 mg/kg prednisolone, the surviving fraction of sensitive cells at 72 hr was 0.05, comparable to that obtained with prednimustine alone. More extensive data for the resistant line are shown in Fig. 3. Combination treatments were adopted in an attempt to enhance the antitumour effects of prednimustine against resistant cells. Injection of chlorambucil (30 mg/kg) 4 hr after prednimustine (60 mg/kg) resulted in the same

Table 2. Toxicity of prednimustine, chlorambucil and binary combinations of chlorambucil and prednisolone in BALB/C mice

Drug	Dose mg/kg [a]	Day of death [b]	21 day survivors
Chlorambucil	2	0	10
	4	0	10
	8	0	10
	16	0	10
	32	0	10
	64	0	10
	128	2^2, 6^2, 10^1	5
Prednimustine	4	0	10
	8	0	10
	16	0	10
	32	0	10
	64	0	10
	128	8^2, 9^1	7
	256	2^1, 5^2, 7^1, 8^2, 9^3	1
Chlorambucil + prednisolone (simultaneously)	2	0	10
	4	0	10
	8	0	10
	16	2^1	9
	32	0	10
	64	2^5	5
	128	2^5, 9^1	4
Chlorambucil followed 4hr later by the same dose of prednisolone	64	0	10

[a] All drugs were injected subcutaneously.
[b] Superscripts indicate numbers of animals dying on day shown. Each group contained 10 animals

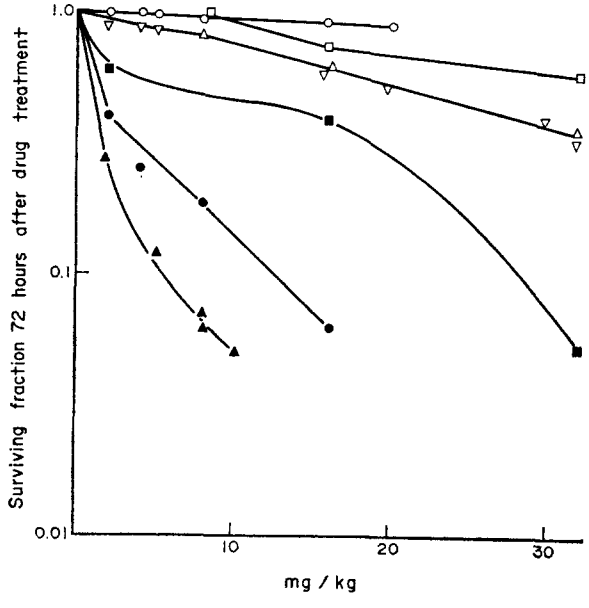

Fig. 3. Survival curves for Yoshida sarcoma cells growing in Wistar rats treated with prednimustine or equivalent (molar) doses of chlorambucil or prednisolone.

Abscissa, mg/kg, *given for chlorambucil, prednisolone or the molar equivalent of prednimustine.*

	Sensitive Cells	*Resistant Cells*
Prednimustine:	▲——▲	△——△
Chlorambucil:	●——●	○——○
Prednisolone:	■——■	□——□
Chlorambucil, 4 hr *later prednisolone:*		▽——▽

At least 3 animals were used at each point and the data shown are the mean of 2 separate experiments. Overall scatter ≯ ± 6%.

cell survival as prednimustine (60 mg/kg) alone, but when prednisolone (30 mg/kg) was given 4 hr after prednimustine (60 mg/kg) the surviving fraction was reduced significantly from 0.32 to 0.23.

Acquired resistance has been developed in the Yoshida tumour strain which fails to respond to alkylating agents [20]. On the other hand the Ehrlich tumour is intrinsically resistant to alkylating agents and was used in the present study to investigate whether prednimustine might be effective against neoplasms which are refractory to chemotherapy *ab initio*. Even toxic doses of prednimustine (128 mg/kg), chlorambucil (64 mg/kg) or combinations of chlorambucil (32 mg/kg) plus prednisolone (32 mg/kg) failed to suppress the growth of this tumour.

Effects of prednimustine on macromolecular synthesis

Table 3 shows the changes in cell volumes, DNA, RNA and protein contents of the sensitive Yoshida cell line following treatment of tumour-bearing animals with prednimustine or chlorambucil. Prednimustine induced both an increase in cell volume and imbalanced growth (as demonstrated by the enhanced RNA:DNA and protein:DNA ratios), typical of alkylating agents, though these changes were less than those resulting from chlorambucil treatment. The effects of prednimustine, prednisolone and chlorambucil on the rates of DNA, RNA and

protein synthesis are shown in Fig. 4. Prednisolone produced an initial fall in the rate of DNA synthesis which returned to the control level after 24 hr. Both prednimustine and chlorambucil induced similar decreases in the rates of synthesis of all the macromolecular species and in this respect prednimustine exhibited properties characteristic of a typical alkylating agent [13]. The incorporation of radioactivity into the acid soluble pools was not altered by prednimustine, prednisolone or chlorambucil, showing that these agents did not restrict the uptake of radiolabelled precursors into the cells.

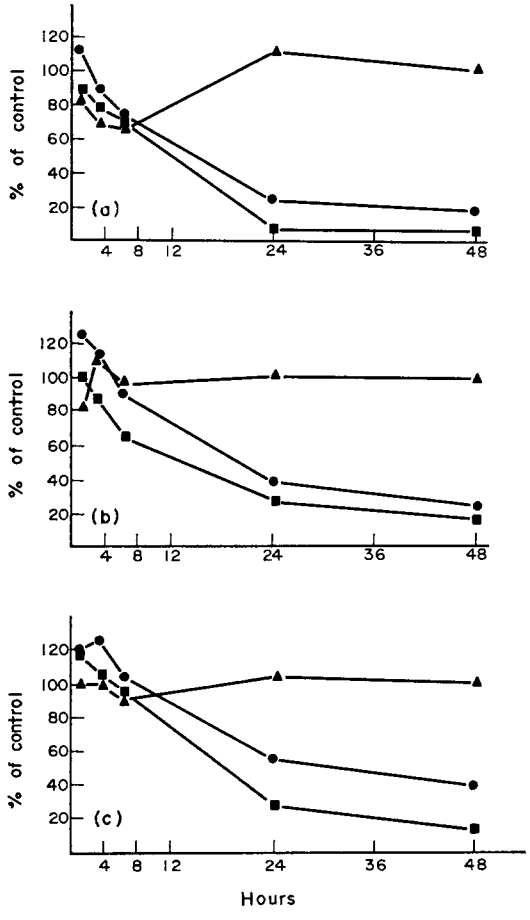

Fig. 4. Rates of synthesis of DNA, RNA, protein in the sensitive line of Yoshida sarcoma cells following treatment of tumour bearing animals with prednisolone, prednimustine or chlorambucil.

(*a*) In vitro *incorporation of* ^{3}H-*thymidine into DNA* (1.0μCi/ml) *for* 15 min, 37°C *following: prednisolone* 10 mg/kg (▲——▲); *prednimustine* 20 mg/kg (●——●); *chlorambucil* 10 mg/kg (■——■).

(*b*) In vitro *incorporation of* ^{3}H-*uridine into RNA* (5μCi/ml) *for* 15 min, 37°C *following: prednisolone* 10 mg/kg (▲——▲); *prednimustine* 20 mg/kg (●——●); *chlorambucil* 10 mg/kg (■——■).

(*c*) In vitro *incorporation of* ^{3}H-*leucine* (5μCi/ml) *for* 15 min, 37°C *following: prednisolone* 10 mg/kg (▲——▲); *prednimustine* 20 mg/kg (●——●); *chlorambucil* 10 mg/kg (■——■).

Cell suspensions were extracted with an equal volume of 0.2N *perchloric acid and RNA, DNA and protein fractionated by the method of Munro and Fleck* [14]. *Each individual point represents the mean from* 3 *animals in* 2 *separate experiments. Overall scatter* ≯ ± 10%.

Role of the prednisolone component of prednimustine

Glucocorticoid hormones are known to inhibit glucose transport [21] and it could be that the antitumour properties of prednimustine derive from such an effect. In order to explore this possibility we have investigated the effects of prednimustine and prednisolone on the transport of 2-deoxyglucose into Yoshida sarcoma cells. Table 4 shows the uptake of [^{3}H]-2-deoxyglucose by alkylating agent sensitive and resistant Yoshida tumour cells following their aspiration from host animals 3 hr after drug administration. It is apparent that neither prednisolone nor prednimustine markedly affected glucose transport into cells of either strain. These results were confirmed in an *in vitro* experiment where resistant strain Yoshida tumour cells were incubated with [^{3}H]-2-deoxyglucose in presence of either prednimustine or prednisolone. The results shown in Fig. 5 demonstrate that neither compound modified the uptake of carbohydrate. These results are perhaps not surprising since in other studies we have found that Yoshida cells contain very few glucocorticoid receptor bind-

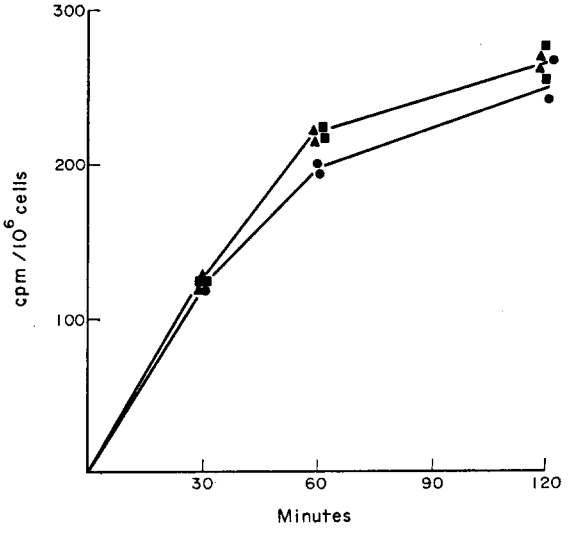

Fig. 5. Uptake of [^{3}H]-2-*deoxyglucose by resistant strain Yoshida sarcoma exposed to prednisolone or prednimustine.*

▲——▲ *control;*
■——■ 10^{-5}M *prednisolone;*
●——● 10^{-5}M *prednimustine.*

Duplicate samples were taken at each point and the results shown represent the mean from 2 *separate experiments. Overall scatter* ≯ ± 5%.

Table 3. Induction of imbalanced growth following prednimustine and chlorambucil treatments

Treatment	Cell volume % of control 24 hr	$\mu g/10^7$ cells at 24 hr				
		DNA	RNA	Protein	RNA / DNA	Protein / DNA
Control	100	146	398	2319	2.73	15.88
Prednimustine 20 mg/kg	126	173	550	3163	3.18	18.28
Chlorambucil 10 mg/kg	177	221	888	4090	4.02	18.51

Animals bearing the sensitive Yoshida tumour were injected subcutaneously with drug 72 hr after receiving 2×10^6 tumour cells.

Each individual point represents the mean from 3 animals in 2 separate experiments. Overall scatter ≯ ± 10%.

ing sites (data not shown). Hence it seemed relevant to explore further the possibility that prednimustine possesses glucocorticoid-like properties. We examined the response of a known glucocorticoid-sensitive lymphoma cell line (S49) to prednimustine and to its individual components. The results are shown in Table 5. It can be seen that both prednimustine and prednisolone, each at similar low molar concentrations, induce comparable amounts of cell lysis. On the other hand the cells are highly refractory to much higher concentrations of chlorambucil.

Table 4. Uptake of 2-deoxyglucose by Yoshida sarcoma cells

Cells	Accumulation of 2-deoxyglucose as % of controls			
	Prednisolone 10 mg/kg		Prednimustine 20 mg/kg	
	30 min	60 min	30 min	60 min
Yosh R	93	78	75	75
Yosh S	141	125	114	108

Control uptake: Yosh R 30 min: 1.62×10^{-9}*
60 min: 1.68×10^{-9}*.
Yosh S 30 min: 1.51×10^{-9}*
60 min: 1.43×10^{-9}*.

*mole/min/10^6 cells.

Tumour bearing animals were injected with prednimustine or prednisolone. After 3 hr the tumour cells were aspirated and incubated *in vitro* in the presence of [^{3}H]-2-deoxyglucose. Replicate samples were taken at each point and the data recorded represents the means of 2 separate experiments. Overall scatter ≯ ± 10%.

Table 5. Effects of prednimustine, prednisolone or chlorambucil on mouse lymphoma cells (S49)

Drug	Concentration	% Cell Lysis
Chlorambucil	10^{-6}M	0
	10^{-5}M	0
Prednisolone	10^{-8}M	0
	5×10^{-8}M	46
	10^{-7}M	69
	5×10^{-7}M	100
Prednimustine	10^{-8}M	15
	5×10^{-8}M	31
	10^{-7}M	43
	5×10^{-7}M	100

Cultures containing 4×10^5 cells/ml were either untreated or exposed to various drug concentrations. The number of viable cells was counted 24 hr later.

DISCUSSION

Prednimustine was less toxic than chlorambucil to Wistar rats, while the simultaneous administration of chlorambucil and prednisolone resulted in toxicity comparable to that produced by chlorambucil alone. However, if the prednisolone component of the combination was given 4 hr after chlorambucil, then the resultant toxicity was significantly less than that produced by chlorambucil alone. BALB/C mice tolerated larger doses of both chlorambucil and prednimustine than did Wistar rats, though in the mouse the former drug proved less toxic than the latter. Unlike the situation with the rat, the simultaneous administration of chlorambucil and prednisolone resulted in greater toxicity than was produced by chlorambucil alone. However, comparable with the

rat, when prednisolone was given 4 hr after chlorambucil, the toxicity of the combination was reduced.

Prednimustine was more effective than chlorambucil in killing an alkylating agent-sensitive strain of the Yoshida tumour. However, it was possible to enhance the antitumour effects of chlorambucil with prednisolone given 4 hr after the alkylating agent, when the tumour cell kill became comparable to that obtained with prednimustine. Prednimustine exerted a significant cytotoxic effect against a Yoshida tumour line which exhibited a 50-fold (acquired) resistance to chlorambucil [12]. Again, however, a binary combination of chlorambucil and prednisolone (the latter given 4 hr after former) produced a tumour cell kill comparable to that of prednimustine. The rationale for the delayed administration of prednisolone derives from previous studies of this laboratory on the whole animal distribution and rates of subcellular binding of chlorambucil, the latter being maximal at about 4 hr [18, 19]. It proved possible to potentiate the antitumour effects of prednimustine with a subsequent dose of prednisolone, though no enhanced antitumour effect was achieved when chlorambucil was given following prednimustine. Hence, since toxicity is reduced and antitumour effects enhanced by delaying the administration of prednisolone when this steroid is used in combination with chlorambucil, it would seem logical to propose this sequence for the combination.

Unlike the two Yoshida tumour lines just discussed, the Ehrlich tumour, which is intrinsically resistant to alkylating agents, proved totally refractory to prednimustine, chlorambucil, or to binary combinations of chlorambucil and prednisolone.

Comparison of the inhibitory effects of prednimustine and chlorambucil on the levels of macromolecules and their rates of biosythesis in drug-sensitive Yoshida cells, indicated that both compounds produced an increase in cell volume and an imbalance in the proportions of RNA:DNA and protein:DNA. These effects are characteristic of alkylating agents, and in these respects prednimustine exerted effects typical of this class of compound [13, 20].

Since glucocorticoids inhibit the cellular transport of glucose [21], it might be argued that the molecular basis of the antitumour effects elicited by prednimustine is inhibition by its steroid component of glucose transport. However, glucose transport into Yoshida cells was insensitive to either prednisolone or to prednimustine. The very low number of specific glucocorticoid receptor binding sites in these cells could account for this result [unpublished observations]. On the other hand prednimustine exerts a cytotoxicity comparable with prednisolone in a steroid-sensitive lymphoma cell line (S49) which is resistant to chlorambucil. It is apparent therefore that prednimustine is capable of exerting a steroid-type function in cells which possess receptor-binding capacity, while not exhibiting such properties in cells possessing few binding receptors. Whether the alkylating and hormonal properties of prednimustine are dependent upon hydrolysis to its component moieties is the subject of present enquiry.

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Effects of Regional Lymphadenectomy and Adjuvant Chemotherapy on Metastasis and Survival in Rodent Tumour Models*

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Abstract—*In view of contradictory reports regarding detrimental effects of regional lymph node removal in animal tumour models, studies were performed to evaluate this controversy. In the experiments it was investigated whether removal of regional lymph nodes (R.L.N.) at any stage of tumour growth had a favourable or unfavourable effect on survival after removal of primary tumours. Removal of tumours was performed also at a stage when early metastases had taken place; this implied leaving a low tumour burden which could possibly be influenced by the host's immunological system. In three tumour models which differed in antigenicity there was no significant effect of R.L.N. removal on survival. In two tumour models which consistently showed lymph node metastases if left untreated, a significant reduction in regional recurrences was noted when regional lymph nodes were removed. In the most appropriate model of the three, the non-antigenic mammary carcinoma, a very pronounced reduction in lymph node metastases was observed when adjuvant combination chemotherapy was given after tumour removal. This was even the case at a level of drug treatment which was ineffective in prolonging survival. In this combined approach, more or less extensive surgery had no discernable effect on survival. Results are discussed in relation to relevance for the clinical management of R.L.N. in breast cancer, with special emphasis on the hazards of regional recurrence.*

INTRODUCTION

THE CONTROVERSY existing regarding the best local treatment for breast cancer is, at least in part, the consequence of the changed concepts which resulted from new information relative to tumour biology. In the field of cancer dissemination, animal experimentation aims at a better understanding of the course of the disease in the human patient. At the present time, this has a particular bearing on the role of the regional lymph nodes draining a tumour. Some experimental studies [1–9] have shown that, at certain stages in the development of a tumour, the regional lymph nodes respond to it and that these early responses appear to be, at least temporarily, inimical to the tumour's progressive growth or resulting metastases. The implications of these observations may be important for the management of uninvolved regional lymph nodes in patients with breast cancer and discussion continues on the merits of retaining or ablating the draining nodes at certain stages in the development of cancer of the breast. In this controversy, one essential condition for valid experimental research is the use of a model which shows similar essential characteristics to the human cancer to which it refers. Even if there is no perfect animal tumour model for any human cancer, certain principles do carry over. The essential characteristics needed for our study include: staging, metastasizing pattern, i.e. lymphogenous and haematogenous spread, similarities in response to therapy and low antigenicity, as expected for

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spontaneous tumours [10]. For the experiments a rodent mammary carcinoma (2661) which shows these characteristics, was selected [11]. Although this model was shown to have essential metastasizing and therapy-response characteristics, a single animal tumour model can only serve as a model for a certain class of tumours found within a disease. Therefore, two additional tumours were evaluated in a similar way. Since most of the tumour models explored to evaluate an effect of removal of regional lymph nodes were antigenic tumours [1–3, 5–9, 12–19] one moderately and one highly antigenic tumour were additionally used.

In our experimental design, we wanted to evaluate whether there was at any stage before or after inoculation of the tumours a favourable or unfavourable effect of regional lymphadenectomy on survival and tumour growth in the area of the regional lymph node. Tumours were removed at a stage when early metastasis had taken place. This meant leaving a low tumour burden which could be influenced by the host's immunological system. The implications of variations in surgery for breast cancer treatment cannot be judged without taking into account the effect of adjuvant chemotherapy. This led us to study also the effect of combinations of adjuvant chemotherapy with more or less extensive surgery in the most appropriate model, i.e. the 2661 mammary carcinoma model.

MATERIAL AND METHODS

Tumour–host systems

Three different tumour–host systems were used in the present study:

1. Mammary adenocarcinoma 2661 was selected from a trial testing tumours on lymph node and lung metastases. Only this particular tumour, which had originated spontaneously in a CBA/Rij mouse, consistently showed metastatic growth in popliteal, inguinal, paraaortic, renal and axillary lymph nodes as well as lethal haematogenous metastases in the lungs, if inoculated via the foot pad. Following subcutaneous and intravenous immunity challenge tests it was found to be nonantigenic [11]. For each of the experiments 2×10^5 tumour cells were inoculated into the foot pad of female CBA/Rij mice weighing approx. 20 g.

2. Lewis lung carcinoma, a widely used experimental tumour, arose spontaneously in the lung of a C57BL mouse in 1951 [20]. After foot pad inoculation, it always metastasizes to the lungs but only rarely to lymph nodes. Following similar immunity challenge tests as those described for the mammary carcinoma system it was shown that this tumour is antigenic in its host [21]. For each of the experiments 2.5×10^5 tumour cells were inoculated into the foot pad of female C57BL/Ka mice weighing approx. 20 g.

3. The RD/3 tumour was obtained from Dr. Carr* in 1975, together with the inbred strain of albino rats, in which it was originally induced by injection of dibenzanthracene. This tumour model has been described as a valid experimental model for lymphatic metastases after foot pad inoculation [22–24]. According to results of the migration inhibition test and growth curves in immunized and nonimmunized hosts, this tumour was found to be antigenic [25]. Immunization with 10×10^6 viable tumour cells in the flanks and subsequent removal of tumours resulted in the regression of all growing tumours (after 5×10^6 tumour cells) whereas non immunized sham operated control animals showed exponential growth of the tumour (unpublished observations). Accordingly, this tumour may be described as a highly antigenic neoplasm. For the experiments with this tumour, 5×10^6 tumour cells were inoculated into the foot pad of rats weighing approx. 200 g.

Tumour cell suspensions were prepared by the trypsinization method described by Reinhold [26].

Technique of regional lymphadenectomy and tumour removal

In a previous publication it was described that following Patent Blue Violet injection in a manner similar to tumour cell inoculation, the mouse foot pad's lymphatic drainage is to the popliteal lymph node [11]. In rats a similar observation was made. Removal of the regional, popliteal lymph node was carried out under pentobarbital (60 mg/kg i.p.) anaesthesia after shaving and cleaning the popliteal skin area with alcohol. Control groups always underwent a sham lymphadenectomy designed to mimic the trauma of lymph node excision. This procedure consisted of a similar skin incision in the popliteal area without exposure of the node. Incisions were closed with metal clips, which were removed 10 days later.

Experimental design

Mammary carcinoma 2661. Except where

*For putting this tumour at our disposal, our thanks are due to Dr. I. Carr, Department of Pathology, University and Weston Park Hospital, Sheffield, England.

otherwise indicated tumours were removed by amputation 14 days after inoculation. This interval was chosen intentionally, since it provided a number of deaths from recurrent disease as well as long-term tumour free survivors. The time of regional lymph node removal or sham lymphadenectomy was used as a variable in order to investigate whether presence or absence of R.L.N. before and at any stage after tumour cell inoculation had any discernible effect. In different studies R.L.N. removal was performed 4 days before, 4 or 8 days after tumour inoculation; simultaneously with tumour removal or 6 days later as a secondary procedure. Our major endpoint for evaluation was survival, plotted as the percentage of mice surviving. At autopsy the extent of metastatic spread was recorded and special attention was given to tumour growth in the popliteal area.

Lewis lung carcinoma

Tumour removal was performed 18 days after inoculation. Times of R.L.N. removal or sham lymphadenectomy were, respectively, 4 days before and 4 and 8 days after tumour inoculation and finally simultaneously with amputation. Secondary removal of R.L.N. was not performed for several reasons: 1. in the other models R.L.N. were already infiltrated by tumour at that time and the main purpose was to evaluate if extent of tumour removal showed an effect on survival at that stage. 2. For the role of the R.L.N. this time in the Lewis Lung system would be of no relevance: systemic immunity would be present at that time as haematogenous spread had already taken place.

RD/3 tumour

Time of removal of tumours and treatment of R.L.N. was somewhat different in this system. Amputation was performed 5 days after inoculation and R.L.N treatment was done 3 days before, 1 day after and 3 days after inoculation. Secondary R.L.N. removal was done 8 days after inoculation. These times were chosen earlier than in the other systems because R.L.N. were almost completely replaced by tumour at day 5. This is similar to data described by Carr and McGinty [22].

Chemotherapy as an adjunct to surgery

This study was carried out only in CBA/Rij mice. A similar combination of drugs as described in an earlier publication [11] was used both on established flank tumours and in the adjuvant situation. When given as full dosage the mice received each week:

100 mg/m^2 cyclophosphamide	day 1, 2, 3	i.p.
20 mg/m^2 methotrexate	day 1	s.c.
300 mg/m^2 5-fluorouracil	day 1	i.v.

This schedule was adapted from the clinically used dosages of Bonadonna *et al.* [27–29] on the basis of equal dose per 4 weeks per m^2 body surface [30] and was continued for 4 weeks. Variations in drug doses were used in order to find a level of drug treatment which had an effect on flank tumours comparable to that of C.M.F. in established recurrences in human mammary carcinoma. Subsequently the full and the half dose schedules were employed in the adjuvant situation to determine the effect of these levels of chemotherapy, with and without surgical removal of R.L.N. at the time of amputation.

RESULTS

All mice that died after treatment in the mammary carcinoma model showed almost total replacement of the lungs by metastatic tumour and in addition lymph node metastases in various sites. Other sites of metastatic spread were observed only very rarely (1 ovarian metastasis, 1 brain metastasis and 2 adrenal gland metastases). Evaluation of experiments was done after a long observation period, since late deaths due to recurrent disease were to be expected. Long-term survivors never showed macroscopic or microscopic evidence of tumour, nor was any benign regrowth of removed lymph nodes observed.

Figure 1 shows the survival curves of animals which had their R.L.N. removed or underwent a sham procedure prior to inoculation of the non-antigenic mammary carcinoma. There is no significant difference in percentage surviving animals ($P = 0.89$). It appears however that animals which had their R.L.N. removed have a somewhat longer delay before death. This difference is not significant and can possibly be explained from autopsy records: all sham lymphadenectomized animals always showed large masses of tumour in the popliteal area (sometimes even over 1 g) which could have affected the ultimate date of death. A similar difference is found in most of the groups to be presented. In a separate experiment estimation of lung weights and ^{125}I-(5)-iodo-(2′)-deoxyuridine (0.5 μCi/g of body weight i.p.)—activity in the lungs of similarly treated animals, simultaneously killed, revealed no differences between lymphadenectomy and sham operated animals,

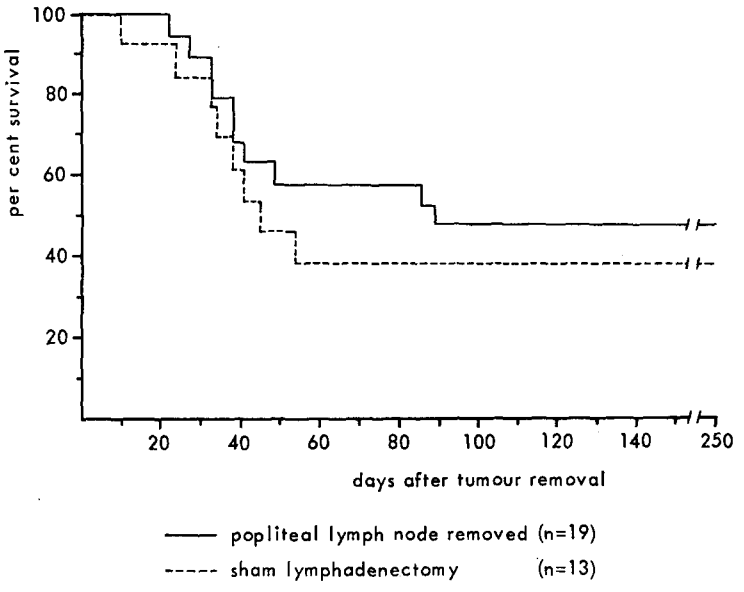

Fig. 1. 2661 *mammary carcinoma. Survival of mice after removal of the primary growth: popliteal lymph node removal or sham lymphadenectomy before inoculation.*

although these parameters seem to be valid indications of the degree of metastatic tumour growth in the lung as shown by much higher lung weights and ^{125}IUdR activity (unpublished observations). If R.L.N. are important in the initiation of immunity it could have been that this specific function, after removal of the R.L.N. prior to inoculation was taken over by other lymph nodes non regional to the foot pad. At days 4 and 8 after inoculation R.L.N. are never infiltrated by metastatic tumour. They are however enlarged due to a reaction to the growing tumour, which has been morphologically interpreted as a sequential combination of a cellular and a humoral immune response [21]. It was shown that an 'early' response consisted mainly of an increase in thymus dependent areas in the lymph node associated with the cellular response. A 'late' response was associated with an increase of the thymus independent humoral response areas. If in the R.L.N. thymus dependent cellular responses are relevant to the rejection of tumour [31] and humoral responses may result in enhancement [32], there should be a difference in 'early' (day 4) or 'late' R.L.N. removal (day 8). Figure 2 shows shows the pooled data of these experiments. Evaluation of the single experiments showed similar results: survival was similar whether treatment of R.L.N. took place at 4 or at 8 days ($P = 1.0$ and $P = 0.74$, respectively).

Removal of R.L.N. at the time of tumour removal could theoretically influence tumour spread, since R.L.N. in most cases contain tumour at this time (14 days after inoculation. Figure 3 shows the survival curves which indicate no difference ($P = 1$) dependent on treatment. It appears that the extent of surgery has no effect on development of blood borne metastases. This finding is similar to that of secondary (tumour containing) lymph node removal at day 20 (Fig. 4). Removal of metastastic lymph nodes independent of whether one (popliteal) or two (also inguinal) lymph nodes were grossly metastatic on palpation at that time after tumour removal had no discernible effect on survival. Nevertheless, the number of palpable metastatic nodes at that time appeared to be a crude index for survival.

As mammary carcinoma 2661 was found to be non-antigenic it could be that findings with tumours which clearly demonstrate antigenicity by influencing tumour cell survival, would differ.

The Lewis lung carcinoma spreads primarily via the blood stream so that this model is less adequate for evaluating recurrence of tumours in lymph nodes. Figure 5 shows the pooled data of experiments similar to those performed in the mammary carcinoma model. Evaluation of experimental groups in which R.L.N. were treated at different times showed no difference; for that reason results were pooled. The surviving fraction of mice is very small in these studies, as a consequence of an earlier haematogenous spread to the lungs. Survival curves run parallel for both groups evaluated.

The RD/3-tumour is a highly antigenic

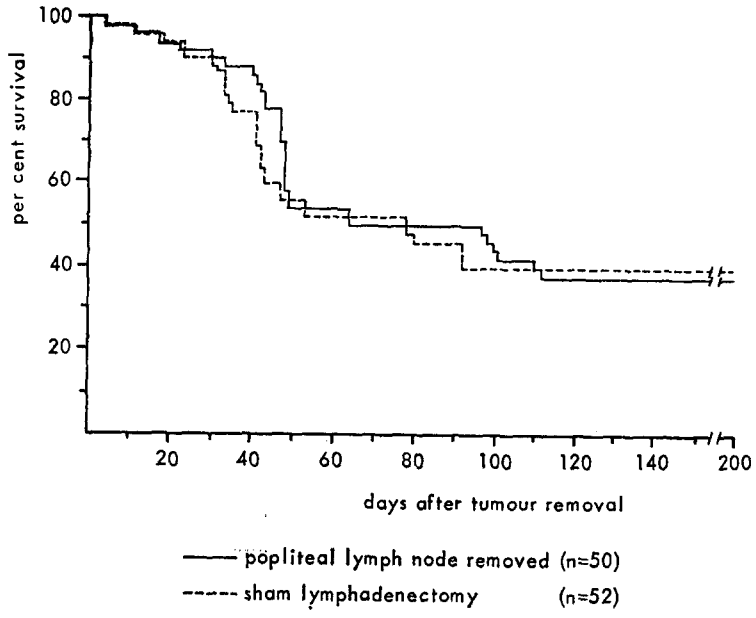

Fig. 2. 2661 *mammary carcinoma. Pooled data on survival after popliteal lymph node removal or sham lymphadenectomy before tumour removal.*

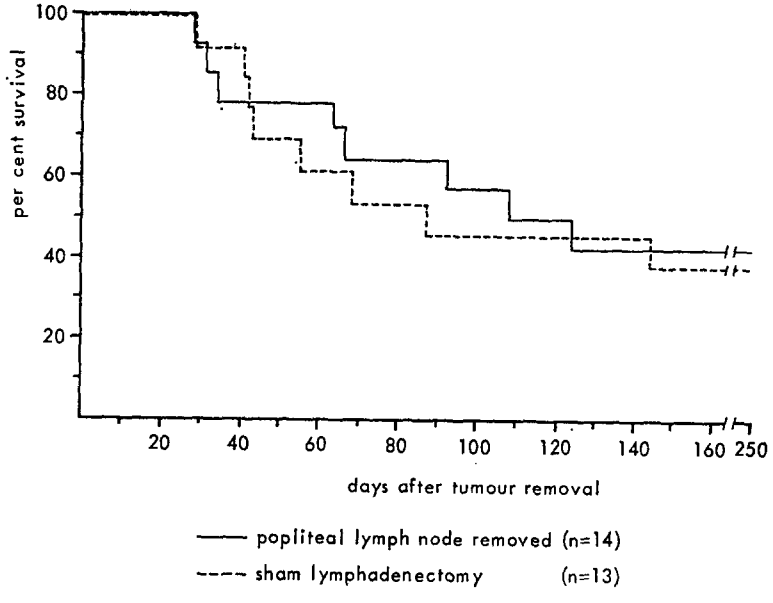

Fig. 3. 2661 *mammary carcinoma. Survival of mice after removal of the primary growth with or without the regional lymph node.*

tumour which metastasizes mainly through R.L.N. In contrast to the findings of Carr and McGinty [22] our rats always died as a result of extensive lymph node metastases in the abdomen. Paraaortic lymph nodes almost completely replaced the abdomen in the absence of any macroscopic or microscopic evidence of tumour cells in the lungs. The reason for this different finding is not clear. The time of R.L.N. treatment differed from that used in the other two models since very early lymph node replacement by tumour was observed, similar to findings of Carr and McGinty [22]. Treatment of R.L.N. was performed before, and 1 and 3 days after tumour inoculation of 5×10^6 tumour cells. A smaller number of 10^6 cells usually produced temporary enlargement of the foot pad which regressed afterwards. For similar reasons as for the Lewis lung carcinoma data were pooled and Fig. 6 shows the results.

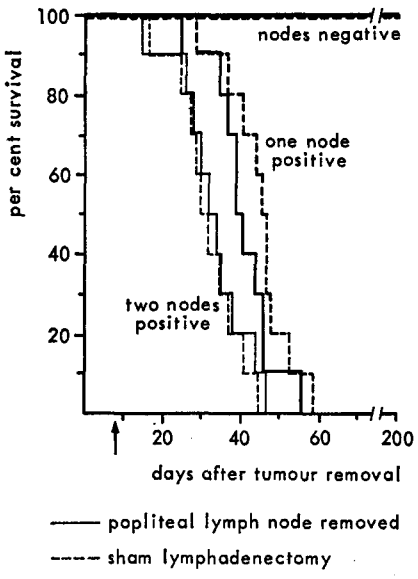

Fig. 4. 2661 mammary carcinoma. Survival of mice dependent on node status and treatment: secondary lymph node removal.

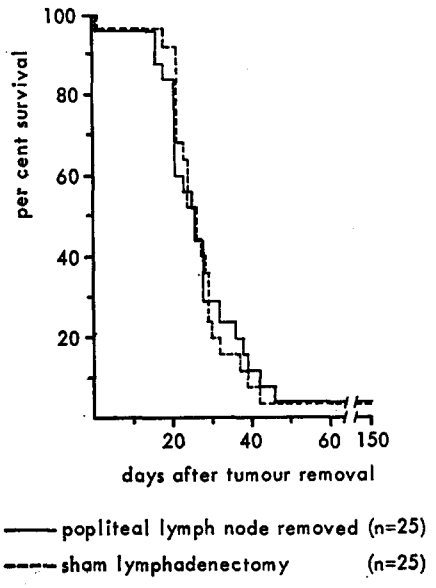

Fig. 5. Lewis Lung carcinoma. Pooled data of experiments comparing removal of the popliteal lymph node with sham lymphadenectomy on survival.

The difference in surviving fraction is not significant ($P = 0{\cdot}46$). A similar phenomenon as found for the mouse mammary carcinoma is observed in the survival curves: i.e. sham-operated animals have a somewhat shorter survival probably due to large tumour masses in the popliteal area.

untreated significant differences were recorded for the incidence of recurrent disease in the popliteal area as shown in Table 2. Recurrent disease was also seen, but in a lower frequency, in animals which had their R.L.N. removed, possibly as a result of spread by lymphatics cut at surgery.

Table 1. *Absence effect of lymphadenectomy on survival**

Time of treatment of R.L.N. 2661 mammary carcinoma	Percentage of mortality from tumour Lymphadenectomy	*P*-values	Sham
Before inoculation	52.6	0.89	61.5
4 days after inoculation	61.3	1	63.6
8 days after inoculation	63.2	0.74	52.6
At amputation	57.1	1	61.5
Secondary	60.6	1	60.6
Pooled data	59.5	1	60.4
Lewis Lung; pooled data	96.0	1	96.0
RD/3 pooled data	47.7	0.46	57.7

*Since staging was not always similar in these experiments, valid comparison is possible only with horizontal groups but not between lines.

As shown in each of the three models evaluated, at any time of treatment of R.L.N. there was no effect in either way. A summary of the surviving fractions in different experiments is presented in Table 1.

However, for the two models which always showed lymph node metastases when left

Adjuvant chemotherapy and R.L.N.-treatment

Figure 7 shows the effects of dosages of adjuvant chemotherapy on established flank tumours. The standard dose refers to the schedule as extrapolated from the clinically applied dosages per m^2 body surface area. As shown, only the full dosage is adequate in

*Table 2. Tumour growth in popliteal area after lymph node removal or sham lymphadenectomy**

Time of treatment of R.L.N. 2661 mammary carcinoma	Percentage of popliteal tumour growth observed in dead animals			Percentage of popliteal tumour growth observed in all animals (including survivors)		
	Lymphadenectomy	*P*-values	Sham	Lymphadenectomy	*P*-values	Sham
1. Before inoculation	20.0	< 0.001°	100	10.5	= 0.007°	61.5
2. 4 days after inoculation	57.9	= 0.020°	100	35.5	= 0.045°	63.6
2. 8 days after inoculation	83.3	= 0.57	100	52.6	= 1	52.6
3. At amputation	20.0	= 0.014°	100	9.0	= 0.023°	61.5
4. Secondary two nodes +	40.0	= 0.011°	100	23.5	= 0.089°	58.8
4. one node +	30.0	= 0.003°	100	18.8	= 0.029°	62.5
Pooled data	42.4	< 0.001°	100	27.4	< 0.001°	60.4
5. Lewis Lung; pooled data	20.0	> 0.10	12.0	—	—	—
6. RD/3-pooled data	66.7	= 0.021°	96.2	31.8	= 0.040°	55.5

*Absolute number of animals in each group are given in the relevant figures (No. 1–6).

P-values counted according to Fisher's exact test for two probabilities (51).

° = significant values.

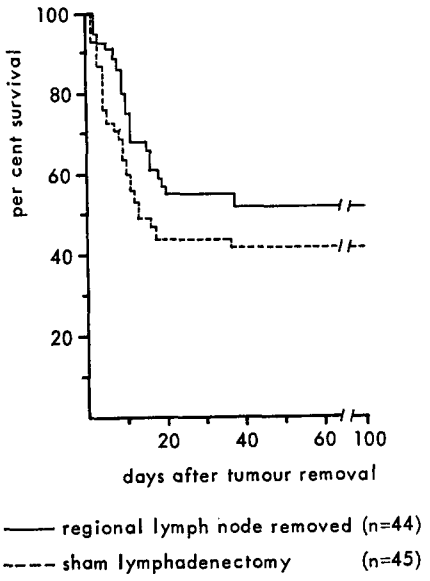

Fig. 6. RD/3 tumour. Pooled data of experiments comparing removal of the popliteal lymph node with sham lymphadenectomy on survival.

producing an objective regression, whereas reduction of dosages resulted in resumption of tumour regrowth during therapy.

Since the size of the primary growth was shown to be a crude index for survival [11] a large tumour was chosen for the adjuvant treatment to determine whether cures could be achieved. Chemotherapy was given at the full and at the half dose level. Surgical removal of the primary tumour with or without simultaneous lymph node ablation took place at 18 days after tumour inoculation; lymph node metastases were already established at this stage. Figure 8 shows the survival curves for treated and untreated animals.

Animals treated by adjuvant chemotherapy consisted of four groups in order to evaluate whether variation in the extent of the removal of tumour mass (i.e. with and without regional nodes) and degree of adjuvant chemotherapy would result in differences in survival. As shown, the full dose schedule results in a significant prolongation of survival ($P < 0.05$). This was not significantly dependent on the extent of surgery. Reduction of the chemotherapy schedule to half dose abolished the effect: survival was similar to controls. The extent of surgery also failed to affect survival significa n this group. A similar difference in the sites of metastases as found earlier (11) was found in the groups treated with adjuvant chemotherapy (Table 3). All animals treated by surgery alone, showed extensive lymph node metastases in addition to lung metastases at autopsy. A significant reduction in numbers of lymph node metastases occurred in the treated groups, in contrast to the much less noticeable effect on the massive lung metastases from which the animals apparently had died.

Even though there was no polongation of survival in the animals receiving only half the dosage, the difference in numbers of lymph

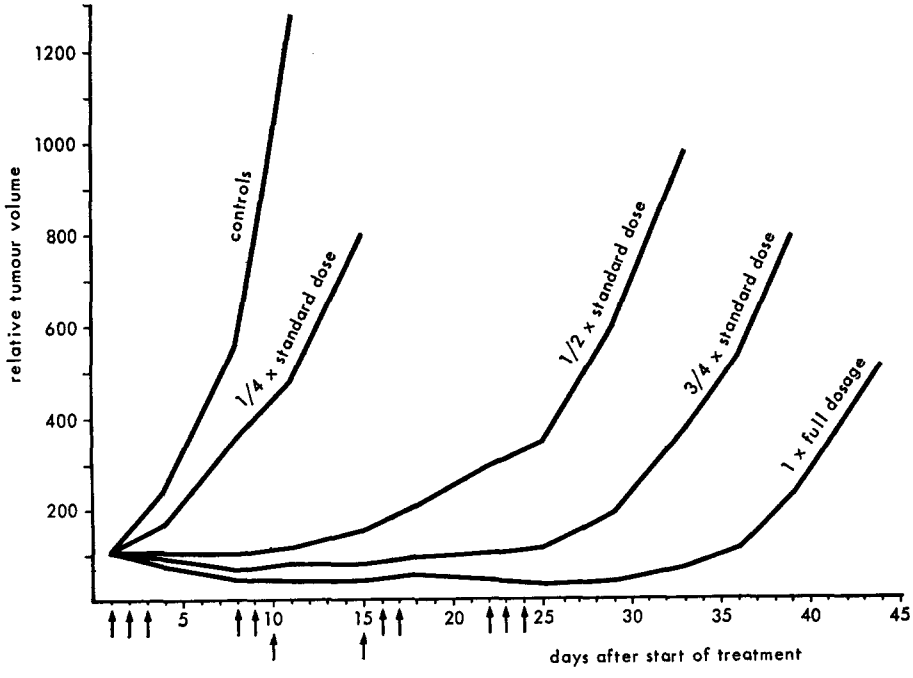

Fig. 7. 2661 *mammary carcinoma. Growth delay of flank tumours dependent on chemotherapy dose.*

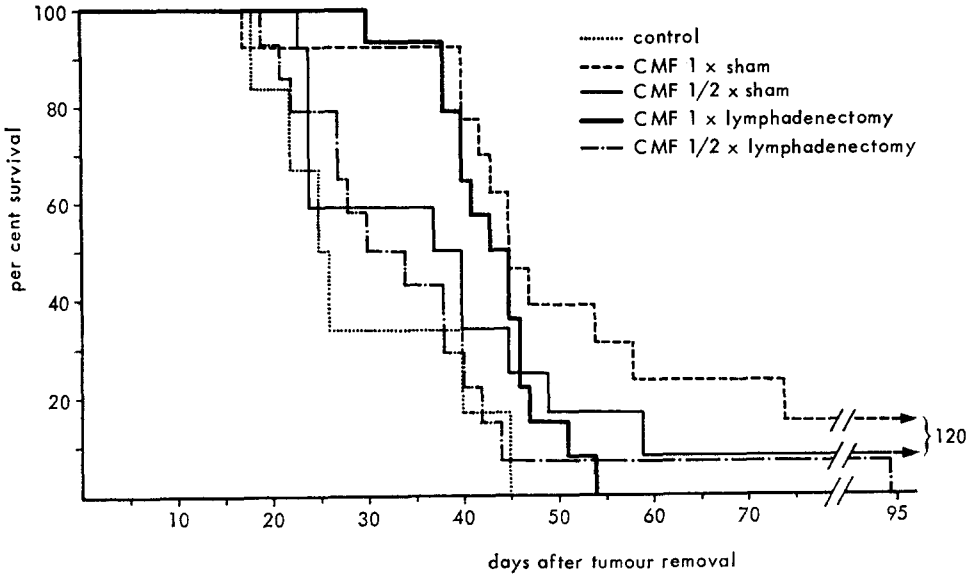

Fig. 8. 2661 *mammary carcinoma. Survival of mice after removal of the primary growth dependent upon lymphadenectomy and chemotherapy.*

Table 3. Percentage of positive nodes dependent upon regional lymphadenectomy and chemotherapy*

Chemotherapy	CMF full dose		CMF half dose		Controls	
Surgery	I.a.	Sham	I.a.	Sham	I.a.	Sham
	3.6%	22.7%	33.9%	50.0%	91.6%	100%

P<0.001
P<0.001
P<0.001
P<0.01
P<0.05
P>0.1
P>0.1

*Four nodal areas were examined. The value presented is the total number of nodes positive/total nodes. The lymph nodes examined were the popliteal, inguinal, paraaortic and axillary on the ipsilateral side with respect to the primary tumour.

node metastases was highly significant. Only in the full dose regimen, a significant difference in the number of lymph node metastases was found between mice treated with lymphadenectomy or not. It must be pointed out, however, that data presented here are expressed as percentages of four nodal areas.

DISCUSSION

In challenging the classical concept of en bloc resection of the primary tumour along with its regional lymphatics for cancer of the breast, Crile's choice of simple mastectomy (i.e. with preservation of R.L.N.) was based on his experimental findings that retention of R.L.N. uninvolved by tumour could be important in maintaining a degree of systemic immunity against growth of metastatic microfoci. Results of his early clinical survival studies seemed to confirm this hypothesis [2, 33–36].

Since then, in addition to the clinical studies, devoted to this controversy, many investigators have developed various experimental models for evaluating the role of R.L.N. in cancer. When reviewing the literature on these studies, differences are noted in tumour models explored, endpoints evaluated and final conclusions. In the present studies, one model, the 2661 mammary carcinoma model, is more fully described, since this is the first model in which an effect on the two modes of spread, i.e. lymphogenous and haematogenous spread, could be evaluated. On the basis of our findings, in both this non-antigenic tumour model and in two additional antigenic tumour models, we must express serious doubts of the unique role ascribed to R.L.N. in antitumour immunity at any stage of tumour growth. On the other hand, relative to the possible barrier function of R.L.N. to tumour cells [37–39] no favourable effect of the removal of R.L.N. which contain tumour was observed in contrast with findings of Sato [12]. An important finding in two models was a significant reduction of tumour growth in the popliteal area. This reduction was observed after R.L.N. removal at any stage. These results indicate that the lymph node itself may present a privileged site in the outgrowth of metastasis. Some experimental data even show that the number of cells necessary to give a 'take' in the lymph node is much smaller than for successful inoculation at other sites [40]. In our studies the large tumour masses in the popliteal area may have influenced survival to a small extent;

similar recurrences in the clinical situation would probably have been treated by radiotherapy and/or chemotherapy, so that this influence would be minimal.

What is the value of these findings among the large list of contradictory reports of experimental studies? Are these models appropriate for conclusions that R.L.N. removal cannot affect ultimate survival?

The presence or absence of some degree of immunological reactivity of the host against its tumour is a controversial condition,regarding the relevance to spontaneous cancer in man of a transplanted animal tumour system. When using quantitative morphometric analysis of draining lymph nodes of the nonantigenic mammary carcinoma and the antigenic Lewis Lung carcinoma we could not find a clear difference in response [21]. It is, however, reasonable to have some doubt about animal tumour systems as models for clinical cancer if they elicit forms of resistance which are clearly attributable to laboratory artefacts associated with their induction. Most of the tumour models previously used for study of the effect of R.L.N. removal involved either highly antigenic, chemically induced, or allogenic tumours. Results from these studies are contradictory: some described a detrimental effect of R.L.N. removal [1–9], whereas others did not find such an effect [13–19, 41] independent of the endpoint evaluated.

Tumours of spontaneous origin were used in only three of the studies: two of them revealed no effect [18, 41], but one showed a detrimental effect of R.L.N. removal in both the initiation [4] as well as in the maintenance of immunity [5]. In these latter studies secondary challenge of tumour was used as an endpoint for the existence of R.L.N. dependent immunity. A reduction in percentage tumours arising at secondary challenge was described in both experimental groups (with and without R.L.N. removal) compared to the first challenge in nonpretreated control animals. This clearly suggests growth affecting antigenicity.

Nevertheless in the present studies antigenicity of the tumour was shown to be of no relevance for the effect of R.L.N. removal. In these studies however we used survival as an endpoint. Many survival curves in the various experiments show 'breaks' before reaching the horizontal level. Skipper [42] observed these breaks in many animal tumour models as well as in human survival curves after surgical treatment. He related these breaks in the slopes of survival curves to the presence of a tumour load of a few or even one tumour cell. If this relation is justified R.L.N. dependent immunity would certainly be expected to have influenced the outcome of an experiment dependent on the survival of such small tumour cell numbers.

From present studies we can qualify R.L.N. only in their properties as part of the reticuloendothelial system's response to tumours. A morphometric analysis of the immune response supports this assumption since it was shown that the reaction of R.L.N. was quantitatively but not qualitatively different from 'distant' lymph nodes evaluated. As a result we cannot support the hypothesis of Crile based on his experimental findings.

The clinical trials comparing simple with radical mastectomy do not confirm Crile's clinical studies. Most of the preliminary reports show no difference in survival [43–45]. This could also be interpreted as evidence that less extensive surgery is equally good. From an emotional and aesthetic point of view, this would be a major improvement. Great caution however must be exercised in this regard: preliminary reports of an international multicenter trial comparing simple mastectomy with radiotherapy to simple mastectomy alone, did show, independent of survival, a significant increase in axillary nodal metastases when left untreated [46]. The 10 years report of a clinical trial comparing radical mastectomy with wide excision showed a similar difference in patients with uninvolved axillary nodes; when axillary nodes were involved, local recurrence and survival were both significantly worse in the latter regimen [47]. Bonadonna noted that local recurrences were decreased by adjuvant chemotherapy [28]. Therefore, we applied this combination of drugs at different levels. Since many experimental studies have shown that there is a inverse relationship between the number of viable tumour cells present at initiation of chemotherapy and curability [48, 49], the comparison of more or less extensive surgery was included. Our findings showed however that there was no relation between extensiveness of surgery and survival. This could be due to the fact that the stage at which amputation was performed was too late. There was apparently a large tumour cell load. A significant reduction in lymph node metastases in contrast to haemotogenous metastases was observed after chemotherapy. This was even the case at a level of drug treatment which was ineffective in prolonging survival. Van Putten *et al.* [50] observed a similar specific decrease of lymph node metastases after cytotoxic drug treatment, associated with an

increase in the number of lung metastases. It is presently not clear whether our finding is due to a difference in cell kill in metastases existing at the time of treatment or to a different localization of regrowth and spread of recurring tumours. The possibility that the lymph bed is a poorer area for regrowth after damage of the lymphatic system by drug treatment cannot be excluded.

Before the general application of less extensive surgery in breast cancer patients is justified by an effect of adjuvant chemotherapy, the presently running trials on adjuvant treatment must be evaluated more fully. Similarly its effectiveness in combination with different surgical approaches should be tested in view of the present findings. Experimental models may share mechanisms and responses to treatment with certain aspects of clinical cancer. In this way employment of suitable models may have a predictive mode and guide the clinician. If experimental results are translatable to man, even if only broadly, it would appear that using such tumour models, the experimentalist is in a position to indicate possibly useful approaches to surgery, adjuvant chemotherapy and the combination of these. Of course such approaches will have to be subjected to the final test of usefulness, the controlled clinical trial. In the evaluation of the results in which survival is the major endpoint, the quality of life closely associated with the incidence of regional recurrences must not be forgotten.

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Letter to the Editor

Cell Killing Effectiveness of Hycanthone*

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IN ADDITION to possessing antischistosomal activity, lucanthone and its metabolite hycanthone [1] have been found to be active against a variety of tumours [2, 3]. These findings made it interesting to determine the activity of hycanthone in our experimental systems consisting of normal haemopoietic stem cells, either resting or in rapid proliferation, an osteosarcoma and leukemia L1210 cells, all in mice.

Hycanthone, as the methanesulfonate salt, was a gift from the Sterling Winthrop Research Institute, Rensselaer, NY. The drug was dissolved in a NaCl solution and injected subcutaneously.

Survival after hycanthone treatment of normal haemopoietic stem cells was determined using the spleen colony assay of Till and McCulloch [4]. Briefly, hycanthone was injected into groups of 5 mice. Sixteen hours later the mice were killed and from their femurs a bone marrow suspension was prepared. The cell suspension was diluted and injected intravenously into groups of lethally irradiated (950 rad of ^{137}Cs γ-rays) mice. Nine days later, these recipients were killed and their spleens fixed in Telleyesniczky's solution. Spleen colonies (each colony arising from one surviving stem cell) were counted. The results were expressed as the number of colony forming units (CFU) per donor femur. CFU survival after treatment was calculated by comparison with the number of femoral CFU from an untreated group. Rapidly proliferating stem cells were obtained by injection of a standard amount of bone marrow cells into lethally irradiated mice. Seven days later their spleens contained sufficient numbers of rapidly proliferating CFU to permit an assay analogous to the method described above.

For the determination of the activity against tumour cells we used the transplantable osteosarcoma C22LR and leukemia L1210 cells, both available in our laboratory in compatible host strains. Drug effectiveness against the solid osteosarcoma was determined by its influence on the relative tumour volume, defined as the product of caliper measurements in three dimensions of the subcutaneously growing tumour [5]. Drug effectiveness against the L1210 leukemia was determined by colony formation *in vitro* after treatment *in vivo* [6]. This was performed by injecting mice intravenously with 10^4 L1210 cells from an *in vitro* culture. After 6 days, groups of 5 of these leukemic mice were treated. Sixteen hours later a femoral bone marrow suspension, containing leukemic cells, was prepared. From this suspension a dilution was made and plated in 35 mm petri dishes with a mixture of Dulbecco's tissue culture medium, 20% serum, 0.3% agar, and 2-mercaptoethanol as a stimulating agent in a final concentration of 60 μmol/l. The dishes were incubated at 37°C in an atmosphere of 10% CO_2 and 100% humidity for 7 days. Colonies consisting of 50 or more cells were counted and the number of leukemic CFU per femur as well as the surviving fraction were calculated by comparison with the untreated control.

Spleen colony assays and osteosarcoma volume measurements were carried out with (C57BL/Rij × CBA/Rij)F_1 male hybrid mice. Survival of leukemia L1210 cells was determined using (C57BL/Rij × DBA_2)F_1 male hybrid mice.

The toxicity of hycanthone in (C57BL/Rij × CBA/Rij)F_1 male mice was determined. A dose of 400 mg/kg caused death within 30 min.

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Two-hundred mg/kg produced immediate morbidity, though the mice recovered soon thereafter, and survived for 30 days. In the assay for normal CFU survival 250 mg/kg appeared to be the highest tolerated dose.

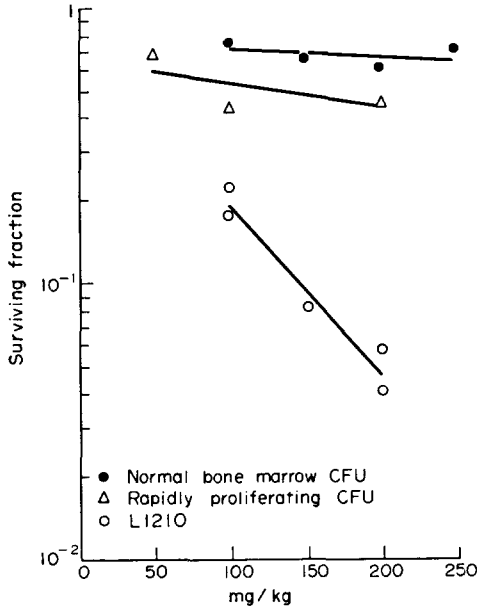

Fig. 1. Dose–effect curves for the survival of resting and rapidly proliferating spleen colony forming haemopoietic stem cells (CFU) and L1210 leukemia cells after subcutaneous administration of hycanthone to mice.

Dose–effect curves for normal resting and proliferating haemopoietic stem cells are presented in Fig. 1. Even at the highest tolerated dose hycanthone was not effective in killing resting stem cells; rapidly proliferating stem cells were more sensitive, but to a minor degree. As a consequence of the ineffectiveness of hycanthone in killing haemopoietic stem cells, no recruitment will take place of resting stem cells into the cell cycle, by which they otherwise should become more sensitive to drug treatment [7]. Since this implies that after prolonged treatment no bone marrow damage will occur, the drug might be suitable for daily administration.

For the osteosarcoma, the tumour volume reduction ratio (TVR) was calculated as the relative volume of the treated over control tumours 4 days after treatment, and a correction was made for differences in tumour volume at the time of treatment. Doses of 125 and 250 mg/kg resulted in TVR values of 0.86 and 0.76 respectively. Earlier observations [5] had made clear that ratio's of 0.5 and higher indicate only temporary arrest of tumour growth. For the osteosarcoma hycanthone clearly belongs to this category.

The dose–survival curve for L1210 cells is presented in Fig. 1. A linear dose–effect relationship was obtained within the dose range tested resulting in a surviving fraction of 5×10^{-2} at the maximum tolerated dose.

The observed absence of bone marrow toxicity of hycanthone and its activity against L1210 cells make it attractive to evaluate effectiveness of hycanthone in combination with other chemotherapeutic agents. However, as hycanthone itself is hepatotoxic [8], combinations with other hepatotoxic drugs must be avoided.

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Perspectives in Cancer Research

Application of the Phenomenon of Changes in the Structuredness of Cytoplasmic Matrix (SCM) in the Diagnosis of Malignant Disorders: a Review*

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Abstract—*A new approach in the detection of human cancer and leukaemias in which changes in the SCM of lymphocytes induced by cancer basic protein (CaBP), phytohaemagglutinin (PHA) and tumour tissue associated antigens are measured with the technique of fluorescence polarization is described. In this review a special emphasis is given to the outline of the effects of experimental conditions on the results of the "SCM test". Normal human lymphocytes* (266/270) *and those from patients with non-malignant disorders* (30/30) *responded to PHA with a decrease in the SCM and gave no response to CaBP, whereas lymphocytes from patients with cancer* (269/272) *responded to CaBP with a decrease in the SCM and gave no response to PHA. The SCM test becomes tumour specific if specific tumour tissues are used for the stimulation of lymphocytes. After successful surgery the lymphocyte responses obtain characteristics of those from healthy donors. Lymphocytes from patients with leukaemia have a subnormal SCM value and gave either an increase or no SCM response to PHA stimulations.*

INTRODUCTION

THE TERM "structuredness of the cytoplasmic matrix" (SCM) is used to describe the physical state of organization of the cytoplasmic matrix at the molecular level. The SCM reflects the forces of interaction between macromolecules, such as proteins and small molecules, such as water molecules, ions, ATP, cyclic-AMP and other cardinal adsorbants [1–3]. Perturbations of these interactions result in changes in the SCM. The SCM is measured with the technique of fluorescence polarization [1]. Fluorescein molecules are introduced into living cells by intracellular hydrolysis of the non-fluorescing fluorescein-diacetate (FDA) and the degree of polarization of the fluorescein fluorescence is determined. The degree of fluorescence polarization is a measure of the SCM. If the degree of fluorescence polarization is high, SCM is high and vice versa.

The "SCM test" for the detection of cancer is based on the phenomenon that the SCM changes during the cell cycle [1]. When cells progress from the resting phase (Go or G1) into the cell cycle the SCM decreases. Responses of human peripheral lymphocytes, which are normally in the Go or G1 phase of their cell cycle, to mitogens and antigens can, therefore, be detected by a decrease in the SCM [4, 5]. We have reported that lymphocytes from healthy donors and donors with non-malignant diseases can be differentiated from lymphocytes of patients with malignant disorders on the basis of changes in the SCM induced by phytohaemagglutinin (PHA), cancer basic proteins (CaBP) and specific tumour tissue associated antigens [4–7]. We are now presenting an up-to-date summary of the results of the "SCM test" obtained on 679 cases. In this review a special emphasis is given to the outline of the effects of experimental conditions on the results of the "SCM test". Details of the preparation and treatment of lymphocytes as well as of the technical aspects in the measurements of fluorescence polarization in living cells are discussed.

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MATERIAL AND METHODS

1. *Preparation of lymphocytes*

1.1. *Collection of blood samples.* Human lymphocytes are prepared from blood collected either in Searle-LH/10 lithium heparin-containing vials or Vacutainer sodium heparin tubes. The heparin concentration should not be less than 300 i.u. per 10 ml of blood. Heparinized blood samples can be stored for up to 24 hr at room temperature without deleterious effects on the SCM responses of lymphocytes.

1.2 *Isolation of SCM-responding lymphocytes.* Ten millilitre samples of blood are transferred into glass vials containing 0.1 g of carbonyl iron powder Type SF (GAF, Great Britain Ltd.) or 8365 w 99.5% iron powder (Koch-Light Laboratories Ltd.) and rotated at 37°C at 20–30 rev/min for 30 min. Vials are then placed on a magnet for 10 min. Lymphocytes are separated by the Ficoll–Triosil gradient technique [8], but using a modified gradient. The gradient is prepared by mixing 24 vol of 9% (by weight) of Ficoll (Pharmacia AB) solution (filtered through a Millipore paper, 0.22 μ), and 10 vol of 35.6% (by weight) of Triosil 440 (Nyegaard and Co. AS, Oslo). The Ficoll–Triosil solution can be prepared in larger amounts, e.g. 1000 ml and stored in a dark bottle at 4°C for up to 2 months. The density of the Ficoll–Triosil solution should be 1.081 g/cm^3 at 25°C and its osmolality 0.320 Osm/kg. The solutions are prepared by using sterile water for injections without preservatives (Phoenix Pharmaceuticals Ltd., Cardiff, Wales, U.K.). Five millilitre aliquots of blood, cooled to 25°C are layered on 5 ml of the Ficoll–Triosil solution equilibrated to 25°C, in 16 mm o.d. centrifuge tubes which are kept in a water bath at 25°C. The centrifuge tubes are wiped dry and centrifuged in a thermostatically controlled centrifuge at 25°C for 20 min at 550 ***g*** (calculated for the interphase position). Only lymphocytes which float on the Ficoll–Triosil solution are collected with a sterile Pasteur pipette, avoiding excess of the blood plasma. None of the lymphocytes which separate inside the Ficoll–Triosil solution should be collected. The latter are SCM-non-responding lymphocytes. Lymphocytes from two gradients of the same donor are pooled into a round-bottom 10 ml glass centrifuge tube and washed twice by adding 6–7 ml of 0.9% preservative-free sodium chloride solution for injections (Phoenix Pharmaceuticals Ltd., Cardiff, Wales, U.K.). Cells are washed by gently drawing them in and out with a clean, sterile Pasteur pipette. Between washes cells are centrifuged at 400 ***g*** for 6 min and the supernatant is decanted. Then lymphocytes are washed once or twice in the same way with complete Dulbecco's [9] phosphate buffered saline (PBS). Lymphocytes from the same donor are pooled and resuspended in PBS in a sterile glass tube at the concentration of not more than 6×10^6 lymphocytes per ml. The suspension is kept in an incubator at 37°C.

The differential cell counts showed that the cell suspensions are over 90% pure lymphocytes. The largest contaminant represent erythrocytes with negligible amounts of granulocytes or platelets. About 25% of the total lymphocyte population is isolated.

1.3 *Stimulation of lymphocytes.* Aliquots of lymphocyte suspensions are incubated at 37°C in sterile glass tubes with phytohaemagglutinin (PHA), cancer basic protein (CaBP) or other antigens for 30–60 min. One tenth of a millilitre of the reconstituted and five times diluted Reagent Grade PHA (Wellcome Ltd.) is added with a 1 ml syringe per ml of lymphocyte suspension mixed by syringing 2–3 times and placed into the incubator. Within the range of undiluted to 20 times diluted PHA the effect is the same. However, we noticed that for some batches of PHA the decrease in the SCM response is not as fast as initially reported [5] and up to 45 min of incubation may be needed to reach the full response. Similarly, the concentration of CaBP is adjusted so that again 0.1 ml is added to 1 ml of cell suspension. Each batch of CaBP has to be assessed for its activity. The CaBP exhibits a sigmoidal dose–response curve, i.e., when full stimulation has been obtained further increase in the concentration of CaBP does not increase the response. For the stimulation of lymphocytes with histologically defined biopsies of tumours, tissues are used either fresh or after storage in dry ice or liquid nitrogen. Before use the frozen pieces of tissue are quickly warmed up to room temperature, washed well in PBS and kept until used for stimulation. Aliquots of 0.5 to 1 ml of lymphocyte suspensions are incubated with tumour tissues, pieces of about 10 mm^3, for 10–15 min at 37°C. To achieve frequent contact between lymphocytes and the tissue, the suspension is gently mixed 2–3 times during the incubation.

In the preparation and incubation of lymphocytes great care should be taken that lymphocyte suspensions of different donors are not cross-contaminated as this will result in a decrease in the control value of the SCM induced by mixed lymphocyte reactions [10] and any further stimulations with PHA or CaBP will be either abrogated or diminished.

2. *Measurement of the SCM*

2.1. *Preparation of the FDA solution.* The fluoresceindiacetate (FDA) is only slightly soluble in water. Therefore, the FDA is first dissolved in acetone. A stock solution is prepared by dissolving 25 mg of FDA* (Koch–Light Ltd.) in 5 ml of spectroscopic grade acetone (Eastman-Kodak). This solution is stored at 4°C and should be prepared fresh weekly. The 2.5 μM FDA substrate solution is prepared by injecting 0.02 ml of the FDA stock solution into 100 ml of sterile, complete PBS (Gibco, Cat. No. 404). Immediately after injection the solution should be well agitated to prevent flocculation. The pH of the PBS must be 7.4 and its osmolality 0.330 Osm/kg; each new batch of PBS should be checked and if necessary its pH and osmolality corrected. Slow thermal hydrolysis occurs in the FDA substrate solution at room temperature. If during the experiment the fluorescence background of the FDA substrate solution should increase to over 20% of the recorder span, a fresh substrate solution must be prepared: even so it can be electronically subtracted on the MPF-4 fluorescence spectrophotometer. As can be seen from Fig. 2 the fluorescence background of the FDA substrate solution is normally about 1% of the recorder span.

2.2. *Equipment.* Measurements of the SCM in living cells are carried out on cell suspensions in a fluorescence spectrophotometer equipped with the polarization accessory. A schematic presentation of the experimental set up is shown in Fig. 1. The bulk concentration of the fluorescein in the SCM measurements is of the order of $10^{-9}-10^{-10}$ M and the increase of the intensity of polarized fluorescence is recorded as a function of time. Therefore, only fluorescence spectrophotometers of sufficiently high sensitivity and stability, i.e., which compensate for fluctuations in the intensity of the exciting light, are suitable. Furthermore, as fluorescein excitation and emission polarization spectra in lymphocytes show that changes in the SCM on mitogen or antigen stimulations can be detected only within a narrow wavelength region [11], broad band filter instruments cannot be used in the SCM measurements. In our laboratory we are currently using a grating type Perkin–Elmer MPF-4 fluorescence spectrophotometer of high stability and wavelength resolution. The degree of fluorescein fluorescence polarization in living cells changes by 3%/°C. A thermostatically controlled cuvette-holder is, therefore, essential. The excitation and emission polarizers used in the SCM measurements must have high precision optical surfaces with negligible light scatter and a polarization defect of less than 0.01.

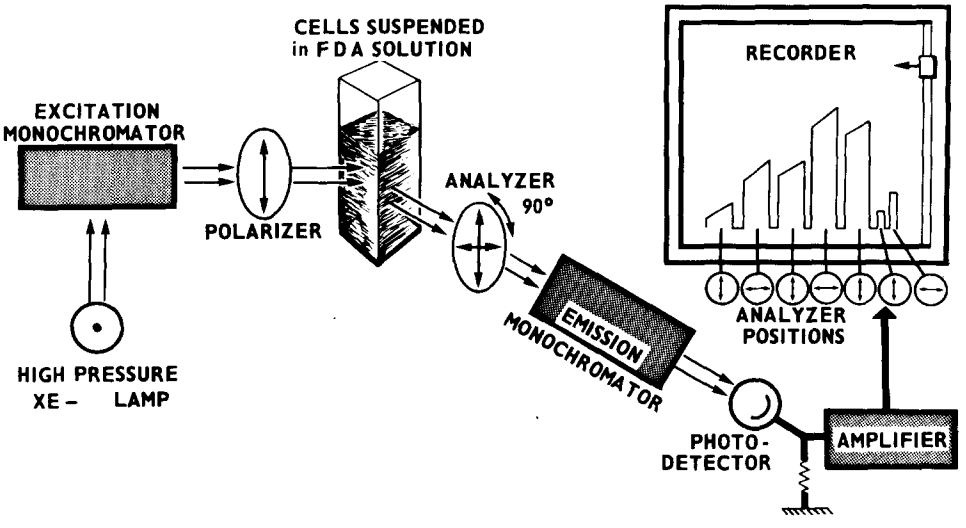

Fig. 1. Diagram of the fluorescence polarisation spectrophotometer arrangement used in the measurements of the SCM on cell suspensions.

*We have noticed that recent batches of acetone, used in the preparation of FDA stock solutions, contain impurities which decrease the SCM of lymphocytes and diminish, or abrogate, mitogenic and antigenic SCM responses. To obviate these effects, the FDA stock solution should be prepared in Aristar grade (BDH) glacial acetic acid. Dissolve 50 mg of FDA in 5 ml of acetic acid by gentle heating, if necessary. This stock solution is stored at room temperature. The FDA substrate solution is prepared as follows: dissolve 0.37 g of $Na_2HPO_4.12H_2O$ in 100 ml of complete, sterile PBS to compensate for the decrease in pH caused by the subsequent addition of acetic acid. The solution must be agitated until all the solid is dissolved to prevent precipitation of calcium phosphates. Inject 0.01 ml of the FDA stock solution into this PBS, shake well and immediately subdilute by injecting 25 ml of this FDA–PBS solution into 75 ml of complete, sterile PBS. The pH of the final substrate solution must be 7.4 and its osmolality 0.330 Osm/kg.

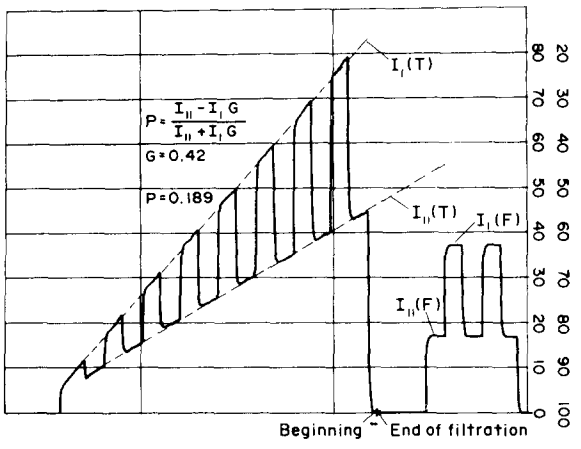

Fig. 2. An example of the SCM recording obtained with a Perkin–Elmer, type MPF-4, fluorescence spectrophotometer.

The polarization defect is defined as $k_2/(k_1+k_2)$, where k_1 is the fractional transmission for polarized light in the pass-axis and k_2 that in the direction perpendicular to the pass-axis [12]. A further requirement is that the polarizers have a low obliquity dependence of the polarization defect, i.e., the polarization defect should not change for incident light angles of up to 30°, and the extinction of light with crossed polarizers should be better than 1%. *In all our experiments we have been using "Polacoat" sheet-polarizers, type 105 u.v. mounted between fused quartz discs (supplied by Perkin–Elmer, U.K.). It is advisable to equip the emission polarizer (analyzer) with an automatic changer of the position as frequent manual changing of the analyzer is tedious and may also result in perturbations of the temperature equilibrium in the cell suspension. Fluorescence polarization measurements on cell suspensions can be affected by artefacts caused by scattered light. This occurs if there is insufficient resolution of the exciting and fluorescent light by the gratings or filters used. It is, therefore, necessary to check the level of scattered light before the equipment is used in SCM measurements. For this purpose 0.2 ml of lymphocytic suspension (6×10^6 cells/ml) is injected into 3 ml of PBS. Using the same settings on the fluorescence spectrophotometer as in the experiment the difference between the signals obtained with parallel polarizers on the lymphocyte suspension and on the PBS solution without cells should be less than 0.5% of the full-scale deflection on the recorder.

2.3. *Experimental procedure.* Aliquots of 0.2 ml of control or stimulated lymphocyte suspensions are slowly injected with a 1 ml syringe into a 10 ml beaker containing 3 ml of the 2.5 μM FDA substrate solution. This suspension is gently swirled and rapidly transferred into a 1 cm quartz cuvette and put into the thermostated cuvette holder of the Perkin–Elmer fluorescence spectrophotometer MPF-4 fitted with the polarization accessory. Measurements are made at 27°C. The excitation monochromator is set at 470 nm (maximum spectral slit-width 20 nm) and the emission monochromator at 510 nm (maximum spectral slit-width 10 nm). At the moment when cells are suspended in the FDA solution, the recorder of the fluorescence spectrophotometer is started. The intensities of the emissions parallel, $I_{\parallel}(T)$ and perpendicular, $I_{\perp}(T)$, to the vertically exciting light beam are recorded alternately (with an automatic polarizer changer) for about 6 min or until $I_{\perp}(T)$ reaches 80–90% of the full scale deflection (see Fig. 2). To correct for the leakage of the fluorescein from the cells and for any fluorescence background in the substrate solution cells are quickly filtered away on Millipore paper (0.22 μ pore size) mounted in a Millipore filter head (Swinex 25 mm dia). The filtration is performed by suction, applying not more than 40 cm of Hg vacuum, using a hand vacuum pump (Mityval Neward Die and MFG, California). The filtrate is collected into a glass tube with a side arm for the vacuum line. Details of the filtration set-up are illustrated in Fig. 3. The times of the beginning and the end of filtration are marked on the chart of the recorder and the fluorescence intensities of the components $I_{\parallel}(F)$ and $I_{\perp}(F)$ in the filtrate (F) are measured (Fig. 2), using the same, meticulously washed, cuvette and orientation of the cuvette as during cell suspension measurements. The fluorescence intensities $I_{\parallel}$ and $I_{\perp}$ emitted from cells are obtained by subtracting values for the filtrate from the total fluorescence intensities $I_{\parallel}(T)$ and $I_{\perp}(T)$ extrapolated to the half-time of filtration. Polarization values, P, are calculated from the relationship: $P=(I_{\parallel}-GI_{\perp})/(I_{\parallel}+GI_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ denote the corrected components of the fluorescence intensities and G the correction factor for unequal transmission of the two components of polarized light. The value of G is calculated from the parallel, $i_{\parallel}$, and antiparallel, $i_{\perp}$, fluorescence intensities emitted from a filtrate solution or 10^{-7}M fluorescein solution in PBS, excited with horizontally polarized light, i.e. $G=i_{\perp}/i_{\parallel}$. For our Perkin–Elmer MPF-4 type instrument $G=0.42$. The reproducibility of polarization values, P, within a series of experiments is better than ±2.5%.

Non-viable cells do not retain fluorescein [13].

*A further requirement is that the polarizers do not exhibit intrinsic fluorescence. For this reason, "Polaroid" visible polarizers cannot be used in the SCM measurements.

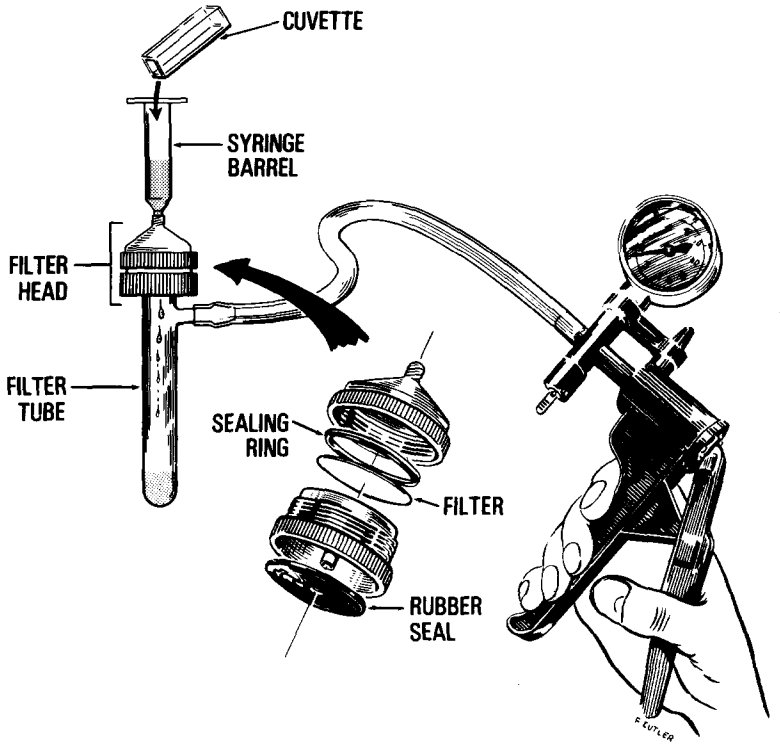

Fig. 3. Details of the filtration set-up.

The contribution of non-viable lymphocytes to the fluorescence intensities $I_{\parallel}$ and $I_{\perp}$ emitted from cells is negligible. The SCM measurements therefore exclude non-viable lymphocytes.

2.4. *Treatment of glassware.* SCM measurements are a high precision microassay which require meticulous control of the fluorescence background. Contamination of filtrates, glassware and cuvettes by fluorescent residues is the most frequent single cause of interference in SCM measurements. Therefore, all the glassware which comes into contact with FDA solutions should be first rinsed with hot tap water, soaked in 50% nitric acid for at least 2 hr, rinsed 10 times in hot tap-water, soaked in double distilled water for at least 4 hr and finally rinsed 10 times in double distilled water before drying in an oven at 110°C. Glassware which was in contact with cell suspensions is after rinsing with hot tap water soaked for 12 hr in 4% "Extran" (B.D.H.) or other surface active agents which do not contain active chlorine. After rinsing 10 times with hot tap water the same washing procedure as that outlined for glassware which was in contact with FDA solutions is followed.

RESULTS AND DISCUSSION

1. *Effects of the experimental conditions*

1.1. *Density of the gradient.* Lymphocytes which respond to PHA and CaBP stimulations in the SCM test are isolated over a narrow range of the Ficoll–Triosil solutions of densities from 1.080 to 1.086 g/cm^3 (Fig. 4). However, our recent results suggest that there is a narrow window at a density of 1.081 g/cm^3 where an enriched SCM-responding lymphocyte population is isolated which on stimulation decrease the polarization to as low as 50% of control values. The density of the Ficoll–Triosil solution, ρ, decreases with increasing temperature according to the following relationship:

$$\rho_T = \rho_{18} - 3.43 \times 10^{-4} \times (T^\circ - 18^\circ), \{g/cm^3\}$$

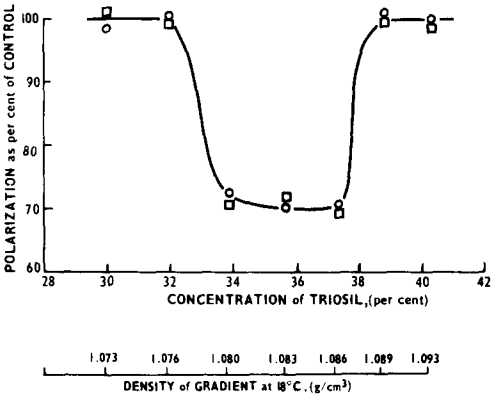

Fig. 4. Effect of the Ficoll–Triosil gradient density on the SCM responses of isolated lymphocytes to PHA (circles) or CaBP (squares).

where ρ_T and ρ_{18} denote densities at T°C and 18°C, respectively. This equation can be used to find the exact temperature at which a Ficoll–Triosil solution will have the density required for the isolation of an enriched SCM-responding lymphocyte population.

SCM-responding lymphocytes are those which float on the Ficoll–Triosil solution. Lymphocytes which band out inside the Ficoll–Triosil solution are SCM non-responding cells which are characterized by a 20% lower SCM value than that of the SCM responders. Dilution of the SCM-responding lymphocytes with non-responders results in progressively lower SCM values and smaller responses on stimulation.

1.2. *The FDA substrate solution.* The SCM values and in part the responses of lymphocytes to stimulation depend on the hydrogen ion concentration (pH), calcium ion concentration, osmolality and temperature of the FDA–PBS substrate solution. The effect of the pH of the substrate solution on the SCM value of lymphocytes and responses to PHA (or CaBP) is shown in Fig. 5. It can be seen that at pH below

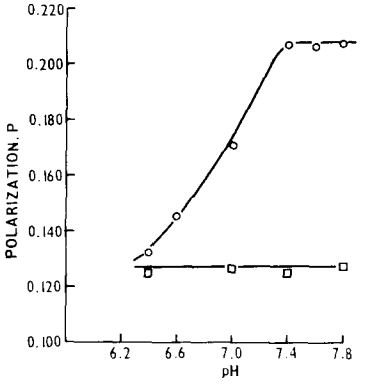

Fig. 5. Effect of the pH of the FDA substrate solution on the SCM values (circles) and PHA (or CaBP) responses (squares) of lymphocytes.

7.4 the fluorescence polarization value, P, (i.e. SCM value) progressively decreases with decreasing pH of the PBS. Since the SCM value of stimulated lymphocytes is pH independent the response to stimulation measured as per cent of the control SCM value decreases. Similarly, when the concentration of calcium ions decreases from 2×10^{-3}M to no calcium ions in the PBS the SCM value of unstimulated lymphocytes sigmoidally decreases by 20% and thereby decreases the per cent of SCM responses to stimulation [3].

The reproducibility of the SCM values depends on the osmolality and temperature of the FDA–PBS substrate solution. With increasing osmolality of the substrate solution the SCM value progressively increases from $P=0.118$ at 0.104 Osm/kg to $P=0.244$ at 0.587 Osm/kg [3]. The osmolality of the PBS should be 0.330 Osm/kg. Variations in the osmolality of up to $\pm 10\%$ will affect the control values of the SCM but not the magnitude of the per cent of SCM changes on stimulation.

As shown in Fig. 6 the temperature of the substrate solution during the SCM measurements affects equally the SCM values of control and stimulated lymphocytes by 3%/°C. We measure the SCM at 27°C. This temperature was selected as a compromise between the rate of FDA hydrolysis and the rate of permeation of the fluorescein from inside cells so that on the average about 80% of the fluorescein is retained in the cells.

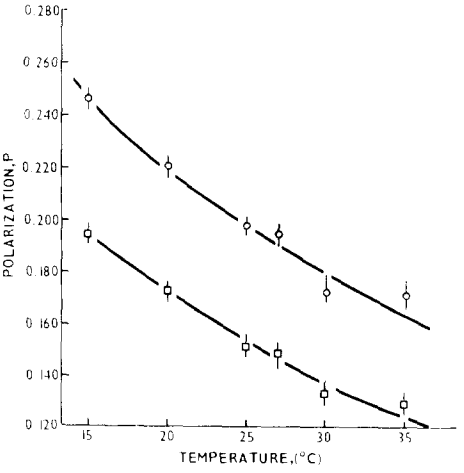

Fig. 6. Effect of the temperature during SCM measurements on SCM values, P, of controls (circles) and stimulated lymphocytes (squares).

1.3. *Wavelength dependence of the SCM changes.* Significant changes in the SCM of lymphocytes in response to stimulations with PHA or CaBP occur in the fluorescein emission polarization spectrum only at 510 nm (Fig. 7). Because of the sharp peak at 510 nm in unstimulated lym-

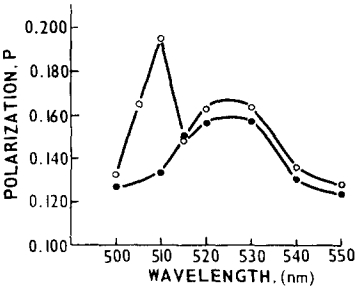

Fig. 7. Fluorescein emission polarization spectra in lymphocytes. Excitation wavelength 470 nm. Controls (open circles), PHA or CaBP stimulated lymphocytes (full circles).

phocytes the spectral slit-width of the emission monochromators, or the wavelength band-width in filter instruments, must not exceed 10 nm. An increase of the spectral slit (or band) width beyond 10 nm will result in a decrease in the control SCM value and increase in the SCM value after stimulation, resulting in a decrease in the magnitude of the SCM responses. As shown in Fig. 8 the degree of fluorescence polarization as measured at 510 nm also depends on the excitation wavelength. The optimal conditions are obtained if the excitation wavelength is set at 470 nm and the spectral slit width (or spectral band width in filter instruments) is 20 nm.

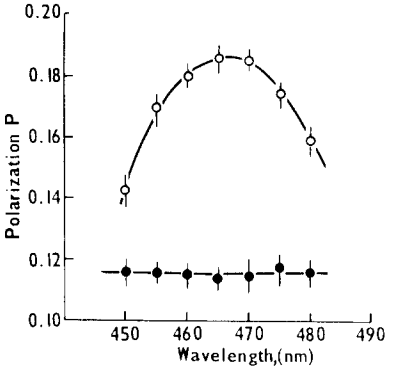

Fig. 8. Fluorescein excitation polarisation spectra in lymphocytes. Emission wavelength 510 nm. Controls (open circles) and PHA stimulated (full circles).

1.4. *Spectrum of the exciting light source.* The cytoplasmic matrix is a heterogeneous and polyphasic system in which the physico-chemical properties, such as pH, dielectric constants and polarity, differ between the micro-domains. Because of this the fluorescein molecule in the cytoplasmic matrix can be in its neutral, cationic and/or anionic molecular structure. These molecular structures have different excitation and emission spectra as well as life-times of the excited state [14]. The fluorescein molecules in the living cells therefore represent an ensemble of different fluorophores, subgroups of which emit photons of the same degree of fluorescence polarization. In this case the average degree of fluorescence polarization measured depends on the fraction of light contributed by each subgroup of fluorophores emitting photons of the same polarization [15]. In the SCM measurements the fluorescein fluorescence is excited with light of 20 nm band-width. The spectral distribution of photons within this band-width can therefore modify the degree of fluorescein fluorescence. Thus, the preferential excitation of fluorescein molecules, which probe domains in which on stimulation changes in the SCM occur, will increase the observed effects. The fluorescein excitation polarization spectrum exhibits a maximal change after stimulations at 470 nm (Fig. 8). The spectrum of the high pressure Xenon light source has a sharp peak at 469 nm and is, therefore, an excellent light source for the SCM measurements. For example, we find that with a tungsten light source the average degree of fluorescence polarization of unstimulated lymphocytes is 80% and the decrease in the SCM observed after stimulation is about half of that obtained with a high pressure Xenon light source.

2. *Summary of the SCM tests*

2.1. *Responses to PHA and CaBP.* The mean value of the SCM of lymphocytes from 270 healthy donors (174 males and 96 females, ages 18–75 yr) is $P=0.196\pm0.004$ (S.E.). The mean SCM value of lymphocytes from 272 patients with malignant diseases is $P=0.195\pm0.003$ (150 females and 122 males, ages 16–90 yr) and that of lymphocytes of 30 patients with non-malignant disorders is $P=0.190\pm0.002$ (15 females and 15 males, ages 18–84 yr). Lymphocytes from healthy donors and patients with non-malignant disorders responded to PHA stimulation with a decrease in the SCM from 20 to 50% of the control value. Stimulation with CaBP resulted in maximal changes of less than 5% in the SCM [5, 6]. In contrast, lymphocytes from donors with cancer did not respond with a significant decrease in the SCM to PHA stimulation. For up to 180 min of incubation, maximal changes in the SCM did not exceed 5% of control values. However, on stimulation with CaBP the SCM decreased from 10 to 50% of the control value [5, 6].

To express the response of the lymphocytes to CaBP and PHA stimulation as a single parameter and to increase the resolution of the SCM test the "SCM response ratio" (RR_{SCM}) is calculated. The RR_{SCM} value is the ratio of the degree of fluorescence polarization obtained after CaBP stimulation, P_{CaBP} over that after PHA stimulation, P_{PHA}, both measured at comparable times after 30–100 min of incubation:

$$RR_{SCM}=P_{CaBP}/P_{PHA}$$

The summary of the results of 621 cases in Table 1 shows that lymphocytes from patients with malignant diseases can be differentiated from the lymphocytes of other donors on the basis of the RR_{SCM} parameter. For healthy donors the mean RR_{SCM} value is 1.46 as compared to 0.76 for patients with malignant disorders.

Table 1. RR_{SCM} values for healthy donors, patients with malignant and non-malignant diseases

Diagnosis	No. of cases	Fraction of cases in RR_{SCM} intervals 0.5–0.9	0.9–1.0	1.0–1.1	1.1–1.9	Mean value RR_{SCM}
Healthy Donors	270	1/270	1/270	2/210	266/270	1.46
"Non-malignant" conditions:						
Sarcoidosis	2				2/2	1.26
Psoriasis	1				1/1	1.30
Thyrotoxicosis	2				2/2	1.26
Rheumatoid arthritis	2				2/2	1.32
Chronic bronchitis	4				4/4	1.33
Cirrhosis of liver	2				2/2	1.50
Crohn's disease	2				2/2	1.34
Infective hepatitis	1				1/1	1.30
Ulcerative colitis	1				1/1	1.36
Gastric ulcer	1				1/1	1.36
Gallstones	2				2/2	1.31
Multiple sclerosis	5				5/5	1.35
Pregnancy (24–35 weeks)	4				4/4	1.50
Diabetes	1				1/1	1.54
"Benign" conditions:						
Thyroid adenoma	1				1/1	1.25
Verrucae vulgaris	2				2/2	1.32
Lypoma	5				5/5	1.41
Pituitary tumour	2				2/2	1.33
Prostate enlargement	2		1/2		1/2	1.12
Displasia cervix uteri	14	1/14	7/14	6/14		1.00
Histologically benign breast growths	21	8/21	5/21	8/21		0.96
"Pre-malignant" conditions:						
Polyposis coli (familial cancer)	1	1/1				0.78
Hyperkeratosis	1			1/1		1.05
"Malignant" conditions:						
Carcinoma of breast	33	31/33	2/33			0.78
Carcinoma of bladder	13	12/13	1/13			0.77
Carcinoma of tongue	9	8/9	1/9			0.78
Carcinoma of larynx	25	24/25	1/25			0.77
Carcinoma of skin	19	18/19	1/19			0.71
Carcinoma of cervix	31	30/31	1/31			0.78
Carcinoma of lung	26	25/26	1/26			0.79
Carcinoma of uterus	10	10/10				0.78
Carcinoma of colon and rectum	12	11/12	1/12			0.72
Carcinoma of pharynx	3	3/3				0.81
Carcinoma of ovary	23	22/23	1/23			0.78
Carcinoma of kidney	3	3/3				0.69
Carcinoma of oesophagus	8	8/8				0.75
Carcinoma of thyroid	3	3/3				0.76
Carcinoma of pyriform fossa	2	2/2				0.80
Carcinoma of epiglottis	2	2/2				0.85
Carcinoma of prostate	2	2/2				0.79
Carcinoma of tonsils	2	2/2				0.76
Carcinoma of stomach	2	2/2				0.72
Carcinoma of ear	1	1/1				0.76
Carcinoma of mouth	7	6/7	1/7			0.80
Carcinoma of alveolus	4	4/4				0.81
Carcinoma of parotis	1	1/1				0.75
Carcinoma of ethmoid sinus	3	3/3				0.74
Carcinoma of penis	2	1/2			1/2*	*1.26, 0.85
Carcinoma of vulva	1	1/1			post biopsy	0.80
Brain tumours	7	6/7	1/7			0.78
Secondary tumour of neck	1	1/1				0.76
Seminoma	1	1/1				0.85
Bone Ewing's tumour	2	2/2				0.74
Malignant melanoma	4	3/4	1/4			0.76
Osteogenic sarcoma	2	2/2				0.72
Reticulum cell sarcoma	1	1/1				0.75
Myosarcoma (Leiomyosarcoma)	1	1/1				0.80
Fibrosarcoma	2	1/2			1/2	1.17
Mesothelioma (?no histology)	1				1/1	1.10

To illustrate the extent of separation or any overlap between the RR_{SCM} values of cancer patients, healthy donors and patients with non-malignant disorders the RR_{SCM} values are divided into two intervals of 0.1 in the RR_{SCM} region of 1.0 and two further intervals encompassing the maximal spread of the RR_{SCM} values above 1.1 and below 0.9. The results obtained on 270 healthy donors and 30 donors with non-malignant disorders which in other tests for the diagnosis of human cancer gave false positive results [16–18] show that 98% of these cases responded on PHA stimulation with an average decrease of 30% in the SCM value and did not respond to CaBP stimulation, resulting in an average RR_{SCM} of 1.46. Between the healthy donors we found four exceptions. Two cases responded to CaBP with a decrease in the SCM of 23 and 16% and to PHA stimulation with a 19% decrease and no response, respectively, resulting in RR_{SCM} values of 0.96 and 0.84. In further 2 cases the unstimulated lymphocytes had a low SCM value of $P=0.150$ and responded in the first case both to PHA and CaBP with an increase of 30% in the SCM and in the second case no responses to PHA and CaBP were observed. The results of the latter 2 cases are similar to those obtained on lymphocytes from patients with chronic lymphocytic leukaemia and pre-leukaemia, respectively (Table 3). The former case also had a ribosomal RNA molecular weight profile characteristic of chronic lymphocytic leukaemia lymphocytes [20].

Between 272 cancer cases studied are 37 groups of different sites of malignancies. Most of the groups include early, intermediate, and late stages of the disease with metastases (Table 1). 93.4% of all cases responded to CaBP stimulation with an average SCM decrease of 25% and no response to PHA stimulation resulting in an average RR_{SCM} of 0.76. A further 5.9% of the cases responded to CaBP with only a 5–10% decrease in the SCM and no response to PHA. We found 3/272 cases of cancer (1.1%) which gave an RR_{SCM} characteristic for non-malignant disorders, i.e., >1.1. In two cases, i.e., cancer of the penis (post biopsy) and fibrosarcoma the SCM tests were not repeated. Therefore, we do not know if they represent "true false negatives" or can be ascribed to a technical error. In the case of mesothelioma no histology report was available.

In the progression from the normal to "pre-malignant" to malignant state the RR_{SCM} value appears to decrease from values greater than 1 to smaller than 1. For example, in a case of hyperkeratosis of skin lymphocytes responded to PHA and CaBP stimulation with a 15% and 10% decrease in the SCM, respectively, resulting in RR_{SCM} values of 1.05, but fully developed cancers of the skin gave RR_{SCM} values from 0.63 to 0.79. In a case of the familial condition, polyposis coli, the lymphocytes responded to CaBP stimulation with a small (9%) decrease in the SCM; however, on stimulation with PHA the response was a 17% increase, resulting in the RR_{SCM} value of 0.78 (Table 1).

In the group of patients with benign growths, i.e., benign pituitary tumours, lypomas, verrucae vulgaris and thyroid adenoma gave RR_{SCM} values characteristic for non-malignant conditions (Table 1). In 2 cases of enlargement of prostate one gave repetitively a 9% response to CaBP and no response to PHA ($RR_{SCM}=0.91$). No histological data were available for this case. The second case gave a normal RR_{SCM} value of 1.34. In cases of histologically diagnosed displasias of the cervix uteri 1/14 gave a RR_{SCM} value of 0.84, 7/14 gave a border-line (−5%) response to CaBP and no response to PHA and 6/14 gave no response to CaBP, but had a diminished response to PHA. The RR_{SCM} values are similar to those in "pre-malignant" conditions (Table 1). In contrast to displasias, 5/5 cases of cancer *in situ* of the cervix uteri show full responses to CaBP and no responses to PHA with RR_{SCM} values of 0.82. Cases of histologically declared benign growths in the breast gave a distribution of RR_{SCM} values similar to that in displasias of the cervix uteri. As shown in Table 1, in 8/21 cases no response to CaBP and a diminished response to PHA was observed (RR_{SCM} values of 1.0–1.1) and 5/21 cases gave an insignificant response to CaBP (<5%) and no response to PHA (RR_{SCM} 0.9–1.0). These two groups consisted of seven fibroadenomas, four lypomas and one case of an abscess under the nipple. However, in 8/21 cases the RR_{SCM} values were typical for malignant conditions (RR_{SCM} 0.5–0.9). This group consisted of 4 cases of cystic fibroadenoses, three intraduct and duct papillomatas (with or without nipple discharge) and one case of a lypoma consisting of a partly encapsulated adipose tissue. The recovery pattern after operation was in 6/8 of these cases the same as in histologically declared malignant growth [19]. One could therefore, argue that sensitisation of lymphocytes to CaBP may precede histological changes, and that the sensitization to CaBP could indicate cases which may be committed to become histologically recognisable malignancies if the growths were left *in situ*.

From the magnitude, mode and time dependence of the SCM response of lymphocytes to PHA and CaBP we inferred that the same size and type of the lymphocyte population may be

Table 2. Changes in the SCM of lymphocytes induced by tumour and normal tissues

Diagnosis of blood donors	Average SCM as percentage of control and fraction of cases responding to tissues												
	BCC skin (%)	Ca bladder (%)	Normal bladder (%)	Ca kidney (%)	Ca breast (%)	Ca colon (%)	Ca lung (%)	Fatty tissue lung (%)	SCC skin (%)	Ca stomach (%)	Bone Ewing's tumour (%)	Ca ovary (%)	Normal ovary (%)
BCC skin	**79** (2/2)			107 (2/2)	101 (1/1)				100 (1/1)				
SCC bladder	100 (2/2)	**79** (3/3)	100 (3/3)	101 (3/3)	99 (2/2)	100 (2/2)						99 (1/1)	
Ca renal pelvis		80 (1/1)	100.5 (1/1)	109 (1/1)	107 (1/1)								
Ca breast		102 (4/4)	100 (1/1)	100 (3/3)	**75** (4/4)	98 (1/1)	99 (1/1)		100 (2/2)			100 (1/1)	
Ca larynx					101 (1/1)		102 (1/1)	108 (1/1)					
SCC bronchus		99 (1/1)		101 (2/2)	99 (2/2)		**76** (3/3)	102 (2/2)				100 (1/1)	
Ca ovary					99.5 (2/2)		100 (2/2)					73 (4/4)	101 (4/4)
Ca stomach				100 (1/1)	97.5 (1/1)					**54*** (1/1)			
Ca oesophagus							96 (1/1)			85 (1/1)			
Bone Ewing's tumour					100 (2/2)		99 (1/1)				**73** (2/2)		
Healthy donors	98.5 (1/1)	96 (2/2)	100 (2/2)	102.5 (3/3)	99.5 (1/1)	100 (1/1)	100 (3/3)		98 (1/1)		99 (1/1)	100 (2/2)	99.5 (1/1)

*Autologous biopsy

Table 3. SCM values and PHA responses of lymphocytes from donors with pre-leukaemic and leukaemic conditions

Diagnosis	Number of cases	Mean SCM value P	Fraction of cases responding to PHA stimulation as SCM		
			Decrease	Increase	No response
Refractory cytopenia	7	0.203	7/7		
(RC)	3	0.156		2/3	1/3
Refractory cytopenia with an excess of marrow	1	0.221	1/1		
myeloblasts (RCEM)	8	0.153		1/8	7/8
Chronic myelomonocytic leukaemia	5	0.148		2/5	3/5
Chronic myeloblastic leukaemia	3	0.125			3/3
Acute myeloblastic leukaemia	9	0.128			9/9
Chronic lymphocytic leukaemia	22	0.139		22/22	

involved [5, 6]. Our study on single cells [21] confirmed that indeed a large fraction of the isolated lymphocyte populations respond to both PHA and CaBP. In healthy donors about 50% of the isolated lymphocyte population decreases the SCM on PHA stimulation, and in cancer patients about 40% of the isolated lymphocyte population responds to CaBP stimulation. The SCM measurements on single cells also revealed that in healthy donors about 4% of the isolated lymphocytes changes the SCM on CaBP stimulation and in cancer patients about 20% of the isolated cell population responds to PHA. However, changes in the SCM of individual lymphocytes are less than 10% and mean values of the SCM obtained either from single cell measurements or SCM measurement on cell suspensions do not show significant changes to stimulation by CaBP in healthy donors and to that by PHA in cancer patients [21].

2.2. *Specificity of the SCM test.* Cancer basic proteins (CaBP) extracted from a general pool of cancer tissues are recognised by lymphocytes from all patients with different malignant diseases [5, 6, 18]. To reveal the type or site of the tumour, lymphocytes from healthy donors and from donors with different malignant disorders were stimulated with histologically defined biopsies of tumour or normal tissues [7]. In these tests lymphocytes from donors with different malignant diseases decreased the SCM only when brought into contact or "baited" with tumour cells similar to those which they have encountered in the body of the donor. The largest decrease in the SCM was observed when the lymphocytes were stimulated with the autologous tumour tissue. Lymphocytes from healthy donors did not respond to any normal or malignant tissue after up to 60 min of incubation. A summary of the up-to-date results is presented in Table 2.

2.3. *Changes in the RR_{SCM} after surgery and radiotherapy.* Studies on changes in the RR_{SCM} after surgical removal of malignant tissues showed [19] that within 2 weeks after successful surgery the lymphocytes did not respond any longer to CaBP (8/8 cases) and in 7/8 cases responded again to PHA stimulation with a decrease in the SCM, resulting in RR_{SCM} values greater than 1.20. The same change in the RR_{SCM} value was found in a case of an *in situ* cancer of the cervix after coning: the RR_{SCM} value changed from 0.83 before to 1.31 after coning. In 18 cases with residual or recurrent disease the RR_{SCM} values were typical for malignant conditions.

In 11 cases of cancer of the larynx, ST1, there was no significant change in the RR_{SCM} values up to 7 months after radiotherapy. However, more than 2 yr after radiotherapy 1/3 cases did not respond to CaBP and also not to PHA stimulation, resulting in the RR_{SCM} value of 1.00. In 2/3 cases the RR_{SCM} value was greater than 1.20, i.e., indicating SCM responses characteristic of healthy donors.

2.4. *SCM values and PHA responses in leukaemic lymphocytes.* As shown in Section 2.1, normal human lymphocytes have a mean SCM value of $P=0.196$ and they respond to PHA stimulation with a decrease in the SCM. In contrast, lymphocytes from patients with chronic lymphocytic leukaemia have a low SCM value of $P=0.139$ and respond to PHA stimulation by an immediate and with time of incubation progressive increase in the SCM to values similar to those of normal, resting state lymphocytes [4]. Still lower SCM values were found in lymphocytes from patients with chronic—or acute—myeloblastic leukaemias (Table 3). In these cases lymphocytes did not respond to PHA stimulation. Chronic myelomonocytic leukaemias showed also a lowish value of the SCM and on PHA stimulation lymphocytes in some of

the cases (2/5) responded with an increase in the SCM or in others (3/5) with no change in the SCM (Table 3). The SCM values and responses to PHA stimulation were also studied in a group of patients with refractory cytopenia (RC) of one or more cell lines with no other abnormal characteristics of the marrow or blood picture. These patients can have either a benign course of the disease, or if characterized by a modest excess of marrow myeloblasts (RCEM), are regarded as "preleukaemic" or "smouldering leukaemias" [22]. The results in Table 3 show that between the RC cases studied 3/10 had a subnormal SCM value and abnormal PHA responses, the other 7/10 cases had normal SCM values and gave also a normal response on PHA stimulation. In 9 patients with refractory cytopenia, characterized by a modest excess of marrow myeloblasts (RCEM), 1/9 patients had a normal SCM value and a normal response to PHA. In the other 8/9 cases the SCM values were subnormal and either an increase (1/8 cases) or no SCM response (7/8 cases) was observed on PHA stimulation (Table 3). The clinical course in the RC and RCEM group is often indistinguishable until the evolution of overt leukaemia permits a retrospective diagnosis. Therefore, the SCM measurements might be useful in recognition of potentially leukaemic cases [22].

In order to ascertain that the lymphocyte population obtained on the density gradient is the same as that from normal donors, the ratio of T- and B-cell lymphocytes was assessed using the rosetting techniques. A case of the chronic myelomonocytic leukaemia which gave a subnormal SCM value and no responses to PHA stimulation had 67.7% T-cells and 17.2% B-cells and a case from the RC group with a normal SCM value and normal responses to PHA had 63.4% T-cells and 20% B-cells, i.e. both were in the normal range of T- and B-cell populations. The abnormal SCM values and responses seem to be, therefore, a genuine reflection of the disease in the peripheral lymphocytes.

The SCM measurements on at least one third of the 679 cases summarised in this study were performed in a single or double bind way, i.e., the diagnosis was available after the results of the SCM test were known.

In conclusion, the successful accomplishment of the SCM-test and the reproducibility of the results mainly depends: *First*, on the isolation of the SCM-responding lymphocyte population. *Second*, on the technical characteristics of the fluorescence spectrophotometer and of the polarization accessory. These should meet the requirements of the SCM measurements in terms of sensitivity, stability, wavelength resolution, light source, and polarization defect of polarizers. *Third*, on the strict control of the pH, calcium ion concentration and osmolality of the substrate.

The results indicate that the SCM test could become useful as a clinical aid in the detection and prognosis of human cancer and leukaemias. With the present "laboratory technique" two technicians can carry out at most 4 complete SCM tests per day. However, the SCM measurements can be fully automated by adapting the flow-fluorometer technique to fluorescence polarization measurements on single cells. This would increase at least ten times the number of complete tests which could be performed in a day.

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Effect of *In Vitro* Produced Transfer Factor on the Immune Response of Cancer Patients*

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Abstract—*Transfer factor produced* in vitro *by a lymphoblastoid cell line, subsequent to induction with specific TFd obtained from patients with transitional cell carcinoma of the bladder (TCCB) or hypernephroma was used for injection of TCCB or hypernephroma patients. Four patients thus injected showed an increase in their reactivity to allogeneic and/or autologous tumour cells in the leucocyte migration test (LMT). E-rosette counts and Con-A and PHA stimulations were also increased following TFdL injections. The specificity of these reactions, as well as the possible clinical effects and the use of* in vitro *produced transfer factor for cancer therapy, are considered.*

INTRODUCTION

WE HAVE recently reported the *in vitro* replication of specific dialysable Transfer Factor (TFd) for transitional cell carcinoma of the bladder (TCCB) by lymphoblastoid cell lines [1, 2]. The Transfer Factor produced by such a lymphoblastoid cell line (TFdL) was induced with TFd obtained from patients whose leucocytes were found reactive to tumour cells. The leucocyte migration test (LMT) was used in these experiments to assess *in vitro* transfer of reactivity following incubation of unreactive leucocytes with TFdL.

Since several reports have already shown that TFd can be used with beneficial effect for treatment of cancer patients [3–6], we undertook the study of the effect of TFdL injections in four such patients.

We present here further evidence that specific TFd from patients reactive to tumour cells (TCCB and hypernephroma) can be reproduced *in vitro* by our lymphoblastoid cell lines and that it transfers reactivity when it is injected into non-reactive patients. Four patients were thus injected with TFdL and the state of their immune system was followed during 7–11 months. They all responded to the TFdL injections, but the reactivity of their leucocytes varied during the course of this study. Our central experimental criterium in these studies was the acquisition, or the increase, of the reactivity (i.e. inhibition) of the patients' leucocytes in the LMT in presence of tumour cells.

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MATERIAL AND METHODS

Two patients with TCCB stage T2, one patient with hypernephroma and pulmonary metastases, and one patient with TCCB and hypernephroma were studied. Prior and subsequent to each TFdL injection, the patients were tested in the LMT against TCCB or hypernephroma formalin-treated cells [1, 2], depending upon the tumour under study, which were obtained from allogeneic, and, in some instances, the autologous tumours after surgical excision. Lymphocyte counts, E-rosettes and PHA and Con-A stimulations were also studied during this period. E-rosettes were evaluated using the method of Jondal *et al.* [7]. PHA-P was used at 1 μg/ml for 10^6/ml lymphocytes distributed in microplates, each well containing 0.2 ml. Con-A was used at 30 μg/ml under the same conditions.

Patients were injected for the first time with

TFdL from 10^8 or 2×10^8 cells and tested 3 days later. Injections were repeated if necessary until patients became positive in the LMT. They were retested every month and reinjected with various amounts of TFdL (multiples of 10^8 cells) as soon as they became unreactive in the LMT (see Figs. 1–4).

The *in vitro* replication of TFd has been described elsewhere [1, 2, 8]. Briefly, TFd from patients found positive to TCCB formalin-treated cells in the LMT were used for induction of one of our lymphoblastoid cell lines. Sterile TFd from 5×10^6 lymphocytes was added to 100 ml of tissue culture containing 2×10^5 lymphoblastoid cells per ml. The culture was grown to approximately 4 l. by addition of fresh medium as required, and samples of approximately 3×10^9 cells were harvested every week. Dialysates were prepared by the same technique as that used to prepare TFd [9]. The TFdL thus produced was tested, prior to its *in vivo* use, for its pyrogenicity in the limulus test [10]. It should be noted that some preparations, which were found pyrogenic *in vitro*, did not elicit any febrile reaction when injected *in vivo*.

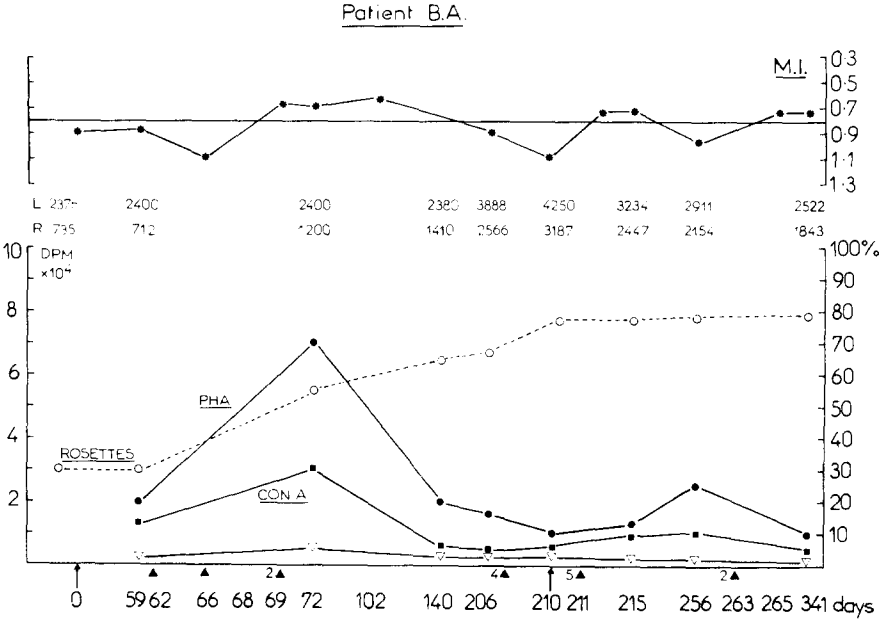

Fig. 1.

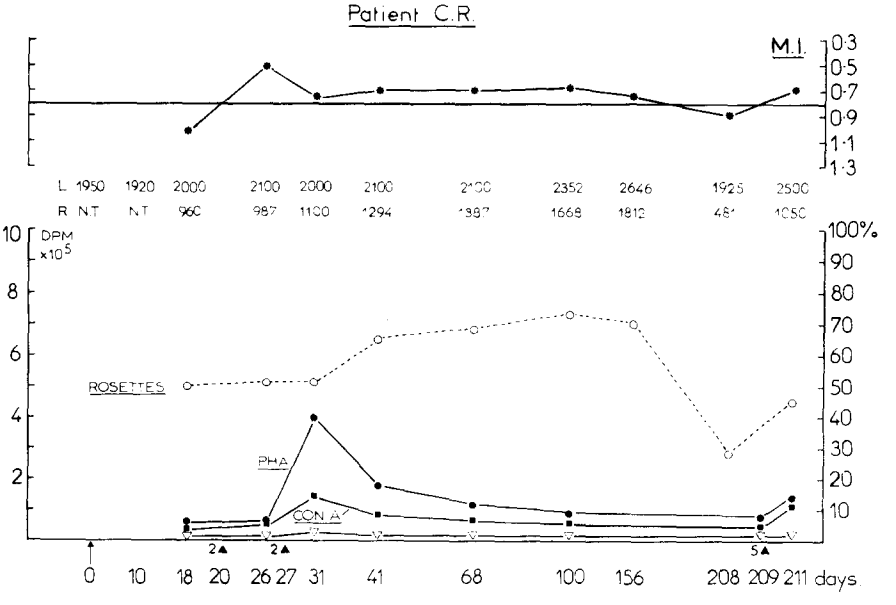

Fig. 2.

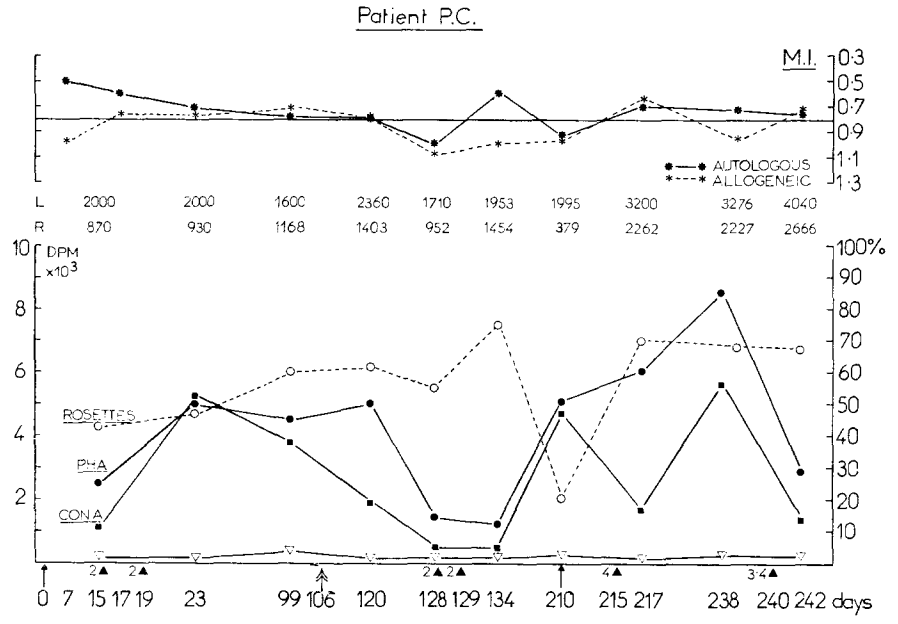

Fig. 3.

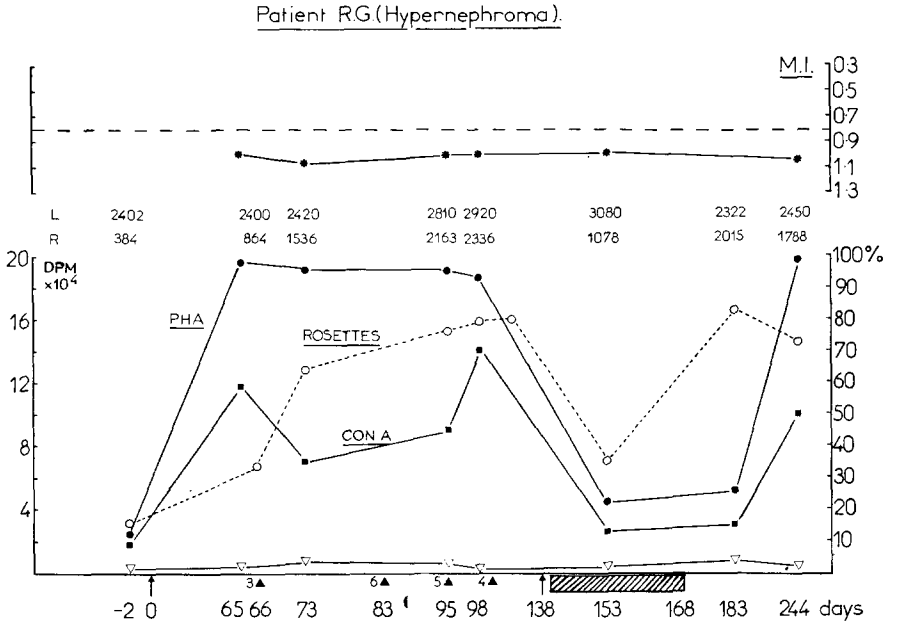

Fig. 4.

Figs. 1–4. *The top of each diagram indicates the reactivity of the patients' leucocytes, with autologous or allogeneic tumour cells in the leucocyte migration test (LMT), during TFdL therapy.* *——* = *Autologous tumour cells;* *– – –* = *Allogeneic tumour cells. (M.I.) Migration Index is significant (positive) if it is below* 0.8. *The second part of each figure indicates PHA, Con-A and E-rosette evaluations during TFdL injections after surgical removal of the primary tumour.*

L = *absolute number/*mm^3 *of peripheral blood lymphocytes.*
R = *lymphocytes/*mm^3 *forming E-rosettes.*
●——● = *Dpm of PHA stimulation.*
■——■ = *Dpm of Con-A stimulation.*
▽——▽ = *Dpm of controls.*
○– – –○ = *E-rosette count* (%).
↑ = *surgery or resection in case of relapse.*
▲ = *injection of TFdL from* 10^8 *cells.*
↟ = *removal of hypernephroma tumour* (*Fig.* 3).
Shaded area = *M.A.P. therapy* (*Fig.* 4).

The activity of the TFdL was checked in the LMT in the presence of the tumour cells from the patients to be injected [1, 2]. It is worth noting here that the TFdL used for injection of patients were induced by allogeneic TFd obtained from reactive TCCB patients. TFdL induced using autologous TFd has not yet been injected. TFdL from 10^8 to 6×10^8 cells was used for each injection (Figs. 1–4).

RESULTS

Case reports

Case 1. (*Fig.* 1). Patient BA is a 63-yr-old female. She was tested in the LMT using her autologous tumour cells one week after surgical removal of her TCCB (stage T2), and she was found negative. She was retested 59 days later with the same result. She received her first injection of TCCB—immune TFdL (10^8 cells) on day 62. Her LMT was still negative on day 65; she then received another injection of TFdL (10^8 cells) on day 66. Conversion of the LMT was observed three days later (d. 69). LMT performed on days 72 and 102 were also found positive. During this period she received one further TFdL injection (2×10^8 cells) on day 69. The patient was found negative when tested 137 days after the last TFdL injection and this coincided with a relapse of the tumour (of 1 mm dia), which was removed. On day 207 she received a TFdL injection from 4×10^8 cells. Three days later she was still negative. A second injection of TFdL (5×10^8) was administered on day 211. Conversion of the LMT was observed 2 days later.

When she was retested $1\frac{1}{2}$ months after this last TFdL injection she was again found to be negative. However, a new administration (2×10^8 cells) converted the LMT within two days and the patient's LMT test remains positive 5 months later.

During the course of the TFdL injections, peripheral blood lymphocytes and E-rosettes were counted and an increase of both was noticed up to day 210, i.e. until the fourth injection of TFdL. The percentage of E-rosettes has remained constant although a decrease has since been noticed in the total number of lymphocytes.

PHA and Con-A stimulations were similarly found to be increased on day 72, i.e. after a total injection of TFdL from 4×10^8 cells. A decrease was subsequently observed, followed by a moderate and transient increase after the TFdL injections. These results are shown in Fig. 1.

Case 2. Patient CR is a 54-yr-old male with TCCB (stage T2) whose reactivity in the LMT against his autologous cells became positive 6 days after the first TFdL injection (2×10^8 cells) administered 20 days after tumour removal. His LMT test remained positive for 5 months, although he received only one more injection of TFdL (2×10^8 cells) on day 27. When the LMT test was found to be negative on day 208, the patient was given TFdL (5×10 cells) on day 209 and his test was found to be positive 2 days later. The LMT remains positive to date, i.e. 150 days after the last injection and 359 days after his operation.

Lymphocyte counts and E-rosettes steadily increased until day 156. Then they decreased. An increase was again observed 2 days after the last TFdL injection on day 209.

PHA and Con-A stimulation increased after the second TFdL injection, but they subsequently fell to the initial values after 2 months. A slight increase was observed after the 3rd TFdL injection (Fig. 2).

Case 3. (*Fig.* 3). Patient PC is a 65-yr-old male. This patient had a double localization of primitive tumours: kidney and bladder. Both his tumours were surgically removed, the TCCB was at stage T2, the hypernephroma at stage T3. The patient was injected using TFdL (2×10^8 cells), specific for TCCB, 15 days after the surgical removal of the bladder tumour. Two days after this injection the patient's cells became positive in the LMT in presence of allogeneic TCCB cells, whereas the reactivity to his autologous TCCB remained positive. A further injection of TFdL (2×10^8 cells) was given 19 days after surgery. The LMT remained positive with both autologous and allogeneic TCCB cells until day 120 when it became negative for both. He then received TFdL from 4×10^8 cells and the LMT was found to be positive 6 days later, but it again became negative after a further $2\frac{1}{2}$ months. At this time a bladder relapse was noted and the tumour was removed. Five days after the tumour resection he again received TFdL (4×10^8 cells). His leucocytes then became reactive for both allogeneic and autologous TCCB cells in the LMT, and this reactivity lasted for 3 weeks. A new injection of TFdL (10^8 cells) again produced a positive result in the LMT with allogeneic tumour cells. It is worth noting that the LMT showed reactivity for allogeneic and autologous tumour cells derived from both the primitive tumour and the relapse. Only a 'one-way' specificity of TFdL was shown in that the TCCB reactivity was transferred but not the hypernephroma re-

activity and this may be considered as being very suggestive evidence for specificity of TFdL.

Lymphocyte counts and E-rosettes increased until the 4th month after surgery and then decreased. This decrease coincided with the relapse. However, they again increased after the 4th TFdL injection given on day 215.

Both PHA and Con-A stimulation increased during the first three months of TFdL injections, but there was a dissociation afterwards, PHA stimulation being restored to a high level whereas Con-A stimulation fluctuated (Fig. 3).

Case 4. (*Fig.* 4). Patient RG is a 38-yr-old male. He had a hypernephroma at stage T2 with two pulmonary metastases of approximately 1 cm dia. Before surgery the patient's cells were reactive in the LMT against allogeneic hypernephroma cells. However, he was afterwards repeatedly tested against the autologous tumour cells and was constantly found negative, and he also lost his reactivity to allogeneic tumour cells 7 months after the operation. During this period he received 4 injections of TFdL, produced with the TFd obtained from another hypernephroma patient, as follows: 1st injection: 3×10^8 cells, 2 months after the operation; 2nd injection: 6×10^8 cells at $2\frac{1}{2}$ months; 3rd injection: 5×10^8 cells at 3 months and the 4th injection of 4×10^8 cells was administered one week after the 3rd injection. *In vitro* tests [7, 10] of the TFdL D_{26}* used for these injections had shown the failure of this TFdL to sensitize the patient's or allogeneic leucocytes to the patient's tumour cells, whereas it was capable of transferring sensitivity towards allogeneic hypernephroma tumour cells, thus strongly suggesting the presence of more than one tumour antigenic specificity in hypernephromas.

E-rosettes increased spontaneously after surgery, whereas lymphocyte counts were not affected. E-rosettes were further increased from 30 to 65% one week after the first TFdL injection, given 2 months after surgery. This increase remained until $3\frac{1}{2}$ months after surgery, i.e. 2 days after the last TFdL injection.

PHA and Con-A responses before TFdL injections were at quite high levels for a cancer patient and no further increase was observed after the TFdL injections.

Since no sensitization to autologous tumour cells in the LMT nor diminution of pulmonary metastases was observed during the TFdL therapy, TFdL injections were discontinued and hormonal therapy was administered (Medroxi-Progesterone-Acetate (M.A.P.) at 1.5 g/day for 30 days). During this therapy the E-rosette counts showed a marked decrease with an increase of the B lymphocytes and the EAC rosettes. The PHA and Con-A responses were also diminished. Two months after the end of the hormonal therapy, E-rosettes, PHA and Con-A resumed their previous values (Fig. 4).

DISCUSSION

For the first time, *in vitro* produced Transfer Factor was injected into cancer patients (TCCB and hypernephroma) who 2 months before the beginning and during the course of the immunotherapy did not receive any other treatment. No side effects whatsoever were noticed during these TFdL injections.

The specificity of the TFdL used was tested *in vitro* in the LMT prior to its injection. Autologous and allogeneic tumour cells were used for these tests. [1, 2]. The TFdL used was always induced by a TFd capable of sensitizing for the patients' tumour cells when tested in the LMT. The TFdL thus derived and injected was, with two exceptions, always active *in vitro* for the patients' tumour cells.

TFdL D_{28} used for the last injection of patients CR and PC (cases 2 and 3) had not shown any activity *in vitro*, but it did show activity when injected, whereas TFdL D_{26}, injected into patient RG (case 4), failed to transfer *in vivo* thus confirming its *in vitro* unreactivity. It was also observed that TFdL A_{12} has a lower activity for *in vitro* transfer and larger doses were needed to obtain an effect *in vivo*, this effect is less marked than with TFdL D_{24} (Table 1) [1, 2]. Since A_{12} and D_{24} are two different batches of TFdL obtained by the induction of two different lymphoblastoid lines using the same inducing TFd, it seems that the various lymphoblastoid cell lines do not respond in the same way to TFd induction. This is now under active investigation.

Specificity of TFdL is clearly suggested in two instances: patient PC who received TFdL specific for TCCB remained unreactive to his hypernephroma cells, whereas he showed a transfer of reactivity to allogeneic TCCB cells. Similarly, patient RG, who received TFdL D_{26}, which never transferred reactivity to his tumour cells *in vitro*, remained unreactive to his tumour cells, whereas he developed

*D_{26} is a batch obtained after a single induction of a lymphoblastoid cell line with a hypernephroma reactive TFd.

Table 1. Leucocyte migration tests (LMT) for patient BA (TCCB). Reactivity of patient BA between d 206 and d 212, i.e. 171 and 177 days after the first TFdL injection. On d 206 the patient did not react to autologous tumour cells (BA) or two allogenic tumour cells (TG and CR), but he did react to the allogenic tumour cells PC. After injection of TFdL from 4×10^8 cells (batch A_{12}) on d 207, no change in the pattern of his reactivity was seen on d 210. The injection of TFdL from 5×10^8 cells, from batch D_{24}, on d 211 resulted in a change of his reactivity when he was tested on d 212. He became reactive to the autologous and allogeneic cells to which he was previously unreactive, and his reactivity to PC tumour cells was increased. Significant LMT inhibitions are underlined. It is worth noting that the patient's leucocytes remained unreactive to tumour cells from two melanoma cell lines (IGR_1 and MEL SK_1). This argues again in favour of the specificity of the TFdL

Date of test		1-IX-75 (d 206)	4-IX-75 (d 210)	8-IX-75 (d 212)
Tumour cells (TC)	Ratio TC: leucocytes	M.I. ± S.D.	M.I. ± S.D.	M.I. ± S.D.
BA	1:200	N.T.	N.T.	1.16 ± 0.01
BA	1:50	1.06 ± 0.02	1.24 ± 0.01	0.89 ± 0.05
BA	1:25	1.00 ± 0.01	1.10 ± 0.01	0.76 ± 0.02
TG	1:50	0.86 ± 0·01	N.T.	N.T.
TG	1:25	0.86 ± 0·01	1.03 ± 0.05	0.79 ± 0.01
PC	1:25	0.65 ± 0·04	N.T.	0.49 ± 0.01
CR	1:25	0.90 ± 0·03	N.T.	0.78 ± 0.02
*IGR_1	1:25	N.T.	1.10 ± 0.01	0.98 ± 0.01
*MEL SK_1	1:25	N.T.	N.T.	1.04 ± 0.02
P.L.	1:25	1.04 ± 0·02	1.06 ± 0.04	0.91 ± 0.03
Date of TFdL injection		4× TFdL A_{12} 2-IX-75 (d 207)	5× TFdL D_{24} 6-IX-75 (d 211)	

*Tumour cells of melanoma lines.
P.L.: Pool of leucocytes from healthy volunteers.
d.: day.
N.T.: Not tested

reactivity to allogeneic tumour cells, thus suggesting the existence of more than one tumour antigenic specificity for hypernephroma tumours. It should be noted here that a possible polymorphism of hypernephroma tumour antigens is consistent with our findings in TCCB [1, 2] and leukaemias [11]. Furthermore, the TFdL injections which induced patient PC's reactivity to allogeneic TCCB cells, never converted his leucocytes to react to his own hypernephroma cells, thus also suggesting specificity of the TFdL and antigenic polymorphism. Also in favour of TFdL specificity is the non-reactivity of patient BA, after TFdL injections, to melanoma tumour cells (Table 1).

TFdL injected into TCCB cancer patients transferred reactivity for their autologous tumour cells in patients BA and CR and for allogeneic tumour TCCB cells in patient PC. In patients BA and CR the reactivity to tumour cells in the LMT transferred with TFdL from 4×10^8 cells was maintained for 4 and 6 months respectively. These data again imply tumour antigenic polymorphism for TCCB, and specificity of the TFdL.

The data obtained by the *in vivo* use of TFdL confirm that TFdL induction is not always successful and also that the activity of the TFdL batches varies from one induction to another. Similarly, a TFdL batch may be capable of transferring reactivity to some antigenic specificities, but not to others, despite their presence in the inducing TFd molecule. It seems that the inducing TFd recruits a population of cells among the cell line for a given specificity, and it is conceivable that the recruitment for another specificity fails or is low in a given experiment.

There are non-specific effects of the TFdL: the increase in the number of T cells and the response to Con-A and PHA, but these effects seem independent of the transfer of reactivity to tumour antigenic specificities. Indeed, they may be obtained when a TFdL induced by TFd from non-cancer patients, is injected into cancer patients with low T cell

counts (Boucheix and Viza unpublished). This non-specific effect of the TFdL on the E-rosettes and the PHA and Con-A stimulations may be compared to an adjuvant effect and it is possibly carried by a different molecule. This is best illustrated by the E-rosette counts of patient RG, which doubled spontaneously within 2 months after operation, and doubled again within one week after the first TFdL injection, whereas his reactivity in the LMT remained low. The increase in the number of lymphocytes and T cells was general for the 3 other patients after TFdL injections, which also induced an increase of the stimulation with Con-A and PHA. However, the increase of the E-rosettes after TFdL injection takes longer to develop than the increase of the response to the mitogens, but the latter is always more transient than the former. Indeed the Con-A and PHA stimulations resumed the low figures within a few weeks, whereas the E-rosettes remained high for longer periods. It should be noted here that preliminary data show that incubation of leucocytes with TFdL *in vitro* does not increase their response to the mitogens. On the other hand, no explanation can be offered for the dissociation between Con-A, PHA stimulations and E-rosette counts observed for patient PC, 7 months after surgery.

For the time being no conclusion can be drawn from these observations showing an *in vitro* increase of reactivity after TFdL injections, as to the value of the use of TFdL in cancer therapy. Only a long-term (5 yr) clinical trial on a large group of patients could prove the clinical efficacy of TFdL.

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Immunoprophylaxis and Immunotherapy of EL4 Lymphoma*

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Abstract—*Injections of appropriate numbers of irradiated, but not iodoacetate treated, EL4 lymphoma cells into C57BL/6J mice were effective in immunoprophylaxis against* 10^6 *EL4 cells and in immunotherapy against* 10^2 *EL4 cells per mouse. The addition of BCG injections made immunotherapy with irradiated EL4 cells effective against a load of* 10^4 *EL4 cells/mouse. Though inoculation of BCG into BALB/c mice inhibited the growth of the allogeneic Ehrlich carcinoma, immunoprophylaxis with BCG only was ineffective against EL4 in C57BL/6J mice. Coating irradiated EL4 cells with concanavalin A did not increase their immunogenicity. Immunotherapy with vibrio cholera neuraminidase and mitomycin treated EL4 cells or with sensitized syngeneic lymphoid cells did not protect EL4 inoculated C57BL/6J mice. However, injections of several rabbit anti-EL4 globulins completely suppressed EL4 lymphoma in a proportion of mice even when treatment was started* 120 hr *after i.p. inoculation of* 10^4 *EL4 cells per mouse. When administered within* 72 hr *of tumour inoculation* ^{131}I *or chlorambucil noncovalently bound to nontumour inhibitory anti-EL4 globulins were at least as effective tumour inhibitors as the inhibitory anti-EL4 globulins.*

INTRODUCTION

INHIBITION of the chemically induced EL4 lymphoma in allogeneic BALB/c and A mice after passive immunotherapy with xenogeneic and allogeneic antisera, enabled Gorer [1] to unequivocally demonstrate specific antigens (i.e. X antigens) on tumour cells. Since then, this tumour has been widely used as a model to assess the effectiveness of various methods of immunoprophylaxis and immunotherapy[2–7].

In an attempt to evaluate the effectiveness of different methods of immunotherapy in this model we have first investigated the lethality of intraperitoneal (i.p.), intravenous (i.v.) or subcutaneous (s.c.) inoculation of varying numbers of viable EL4 cells in the syngeneic C57BL/6J mice and have then re-examined the efficacy of such reported methods of immunoprophylaxis against EL4 lymphoma as peritoneal tapping of ascites tumour bearing mice [3], intradermal inoculation of viable tumour cell [8] and preimmunization with attenuated [9, 10] and antigenically altered EL4 cells [2, 4]. Further, the usefulness of injections of BCG and/or irradiated EL4 cells for immunoprophylaxis was investigated and compared with the results of similar immunomanipulation in an allogeneic tumour model, i.e. Ehrlich ascites carcinoma (EAC) in BALB/c mice, in which the effectiveness of immunoprophylaxis with BCG was initially reported [11]. Finally, the therapeutic response of this tumour to active and adoptive immunotherapy has been compared with the results of immunotherapy with xenogeneic antibody bound to ^{131}I or chlorambucil. Serotherapy with appropriate xenogeneic antitumour globulins (ATG) or ATG bound to cytotoxic agents [5, 7] appears to be more effective in suppressing EL4 lymphoma than the methods of active immunotherapy tested.

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MATERIAL AND METHODS

Tumours

The EL4 lymphoma, which originated in 1945 in a C57BL/6J mouse treated by Gorer [1] with dimethylbenz[a]anthracene, has been maintained since 1946 at The Chester Beatty Research Institute, London, from where we obtained the tumour in 1969 and have main-

tained it by serial i.p. passage in C57BL/6J mice.

Despite the presence of at least three well-defined tumour associated antigens (X, L and E antigens) [6], this tumour is reported to be unhibited by immunoprophylaxis with 'unmodified' or X-irradiated EL4 cells, though allogeneic antibody against the X antigen could protect C57BL/6J mice against inocula of EL4 cells [1].

The Ehrlich Lettre hyperdiploid ascites carcinoma was maintained by serial passage in 12–16 week-old BALB/c mice. Intraperitoneal inoculation of even about 25 viable EAC cells led to solid intraperitoneal tumour formation and subsequent death of tumour inoculated mice. However, 2.5×10^5 viable EAC cells constitute the 'critical inoculum' for the production of ascites in 100% of the mice: the presence of less than this number of viable EAC cells in the peritoneal cavity of mice tends to give rise to solid intraperitoneal tumours [12]. The survival of EAC inoculated BALB/c mice varied inversely with the size of the tumour inoculum. After transplantation into BALB/c mice, EAC grows progressively, invades various organs, forms metastases and kills all tumour inoculated mice in spite of the probable antigeneic differences between EAC cells and the allogeneic host BALB/c mice [12, 13].

Tumour cell and BCG preparations for immunization

Freshly obtained tumour cell suspensions (10^8 cells/ml), after being washed in phosphate buffered saline (PBS, 0.01 M, pH 7.1), were exposed under sterile conditions to 15,000 rad of Y rays from a Gamma Cell 220 ^{60}Co Irradiation Unit (Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada) [7, 12]. Viable BCG organisms (Connaught Medical Research Laboratories, Toronto, Ontario, Canada) were inoculated (10^7 BCG cells/mouse) by various routes as stated in the different experiments.

Neuraminidase treated tumour cells were prepared for immunotherapy following the method of Rios and Simmons [14]. Aliquots of 2×10^6 EL4 cells were incubated with 50 units/ml of vibrio cholera neuraminidase (VCN, General Biochemicals, Chargin Falls, OH, U.S.A.) for 1 hr at 37°C, were washed 3 times with M199 and then reincubated for 1 hr with 50 μg/ml of mitomycin C (Sigma Chemical Co., St. Louis, MO, U.S.A.). The cells were again washed 3 times with M199 and the viability of the cells was confirmed by trypan blue exclusion [5] before injection. Comparison groups of mice were vaccinated with EL4 cells exposed to VCN only, mitomycin C only and M199 only.

The binding of Con A to EL4 cells was verified by autoradiography of EL4 cells [7] incubated with Con A containing tracer doses of ^{3}H Con A (New England Nuclear, Dorval, Quebec, Canada). To coat tumour cells with Con A (Calbiochem, Los Angeles, CA, U.S.A.), 10^7 tumour cells/ml were reacted with 25 μg Con A/ml for 30 min, at 37°C.

Spleen and peritoneal cells for adoptive transfer

Peritoneal cells were collected by washing the peritoneal cavity with Hank's balanced solution (HBSS). Splenic cells were dissociated with 0.2% ethylenediaminotetraacetic acid in PBS and erythrocytes were lysed with 0.83% NH_4Cl. The nucleated spleen cells and peritoneal cells were washed 3 times with sterile HBSS, thei r viability was assessed by trypan blue exclusion and the cells were then suspended in HBSS at the desired concentration.

Anti-EL4 sera

The details of the methods of production and purification of rabbit anti-EL4 sera, the tests to establish their specificity and the method of fractionation to obtain the ATG have been described recently [7, 15]. In brief, groups of female New Zealand white rabbits were intramuscularly injected twice with 4×10^8 EL4 cells mixed with Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) followed by repeated intramuscular injections of 10^8 EL4 cells/rabbit without any adjuvant. The immune sera were rendered tumour specific by repeated absorptions with homogenates of organs from adult C57BL/6J mice until the sera reacted as assayed by immunofluorescence, only with EL4 cells and neither with cryostat sections of normal C57BL/6J mouse tissues nor with suspensions or smears of C57BL/6J lymphoid cells from lymph nodes, spleen and thymus, B16 melanoma cells from C57BL/6J mice and EAC cells. The three batches of EL4 inhibitory rabbit sera were obtained after a total of 12, 22 and 35 injections of EL4 cells. Normal rabbit sera and the anti-EL4 sera after being rendered tumour specific after absorptions with homogenates of normal C57BL/6J mouse tissues were fractionated with 33% saturated ammonium sulphate. The methods of binding chlorambucil [16] and ^{131}I [7, 15] to rabbit globulins have been recently described in detail.

Detection of tumour antibodies

Cytoplasmic and membrane immunofluorescence of mouse tumour cells and other control preparations were carried out by the sand-

wich method using as appropriate fluoresceinated goat antirabbit or rabbit antimouse 7S globulin (Hyland Laboratories, Los Angeles, CA, U.S.A.) [5, 7, 15].

For detection of agglutinating and complement dependent cytotoxic antibodies tumour cells were incubated *in vitro* with immune rabbit or other test sera with and without the the addition of eight times diluted fresh rabbit serum, guinea-pig serum or C57BL/6J mouse serum which were not cytotoxic by themselves and which were assessed for their hemolytic complement activity by the method described by Cinader *et al.* [17]. At the end of the incubation period, the cells were examined for agglutination, morphological changes and permeability to trypan blue [5] and were then injected into syngeneic mice. To detect tumour suppression or enhancement by these antibodies the mean survival of these test mice was compared with that of control groups inoculated with aliquots containing identical numbers of tumour cells incubated with normal rabbit serum or HBBS.

Mice

C57BL/Icrf mice were donated by the Imperial Cancer Research Fund Laboratories, London, U.K. All other inbred mice were purchased from the Jackson Laboratory, Bar Harbor, MN. When not stated otherwise, experimental groups consisted of at least ten 12–16-week-old female mice.

Statistical evaluation

The significance of the difference in mean survival between groups was assessed by Student's *t*-test and the significance of the difference in the numbers of tumour free survivors between groups was evaluated by the fourfold table method of X^2 test.

RESULTS

Survival of C57BL/6J mice inoculated with varying numbers of EL4 lymphoma cells

Table 1 shows that i.p. inoculation of even about 10 EL4 cells leads to progressive tumour formation and subsequent death of tumour inoculated C57BL/6J mice. All the C57BL/6J mice inoculated i.v. or s.c. with 10^3 EL4 cells died approximately at the same time as the mice inoculated i.p. with comparable numbers of EL4 cells. With inocula of 10^7 EL4 cells/mouse, the mice injected i.v. died earlier (survival 9.0 ± 0.7 days) than the mice inoculated i.p. (survival 12.4 ± 0.89 days) or s.c. (survival 12.8 ± 1.1 days). None of the mice inoculated i.v. with EL4 cells had ascites. In the mice inoculated with EL4 cells s.c., ascites was seen only in those mice in which the s.c. tumour had penetrated into the peritoneal cavity. A few of the mice inoculated i.v. with 10^3 or 10^4 EL4 cells had bilateral pleural effusion containing EL4 c

At autopsy, the C57BL/6J mice inoculated i.v. with EL4 cells revealed enlargement of spleen and lymph nodes. With inocula of 10^7 EL4 cells/mouse, the range of splenic weight in the mice inoculated i.v. was 402–508 mg (median, 442 mg), whereas in the groups inoculated with 10^7 cells i.p. or s.c. the range of spleen weights was 65–140 mg (median, 68 mg). The mean spleen weights of 13 normal female C57BL/6J mice of a comparable age group was 70.72 ± 6.25 mg. Immunofluorescence assay using xenogeneic anti-EL4 serum [9] revealed dense accumulation of EL4 cells in the enlarged spleen and lymph nodes from the C57-BL/6J mice inoculated i.v. with EL4 cells. In the C57BL/6J mice inoculated i.p. with 10^8,

Table 1. *Survival of C57BL/J mice inoculated i.p. with varying numbers of EL4 lymphoma cells*

Number of EL4 cells inoculated	Survival (days ± S.D.[a]) After i.p. inoculation
1	—[b]
10	26.6 ± 6.84
10^2	23.4 ± 2.60
10^3	20.0 ± 1.0 21.3 ± 2.91* 20.7 ± 1.2†
10^4	18.4 ± 0.55 18.8 ± 1.64* 19.8 ± 1.48†
10^5	15.6 ± 1.81
10^6	12.8 ± +0.83
10^7	12.4 ± 0.89 9.0 ± 0.71* 12.8 ± 1.1†
10^8	10.0 ± 0.0

[a]Each group contained at least 15 adult female mice.
[b]Only 4 of 42 C57BL/6J mice inoculated i.p. with a single EL4 cell developed tumour.
*Figures represent survival of groups inoculated i.v. with EL4 cells.
†Figures represent survival of groups inoculated s.c. with EL4 cells.

10^7 or 10^6 EL4 cells, the weight of the spleens varied between 60 to 140 mg (median, 71 mg) in contrast to the groups inoculated with 10^5 cells (range of spleen weight 90–230 mg, median 145 mg); 10^4 cells (range of spleen weight 150–240 mg, median 190 mg); 10^3 cells (range of spleen weight 165–190 mg, median 182 mg); 10^2 cells (range of spleen weight 140–310 mg, median 185 mg). It is interesting to note that the volume of ascites fluid at the time of death in the mice inoculated i.p. with $\geqslant 10^6$ EL4 cells was $\geqslant$ 7 ml per mouse, wheras the volume of ascites fluid per mouse in the mice inoculated i.p. with $\leqslant 10^5$ EL4 cells/mouse, did not exceed 4 ml per mouse. The splenic weight of the mice with subcutaneous tumours varied widely and was influenced by the ulceration and infection of the tumours.

Irrespective of the route of tumour inoculation, survival of the tumour inoculated C57BL/6J mice was inversely related to the size of the tumour inocula and histological examination of tissues obtained at autopsy revealed widespread infiltration of EL4 cells into all internal organs.

Effect of peritoneal tapping on the growth of i.p. inoculated EL4 cells in C57BL/6J mice.

There was no difference in the survival between groups of C57BL/6J mice which had their peritoneal cavity aspirated daily for 4 days beginning 24 hr (survival 11.80 ± 1.30 days), 72 hr (survival 12.4 ± 0.54 days) or 10 days (survival 14.6 ± 4.92 days) after i.p. inoculation of 2×10^6 cells/mouse. The survival of comparison groups inoculated i.p. with 2×10^6 EL4 cells/mouse without any treatment or sham aspirated daily for 4 days beginning 24 hr after i.p. inoculation of 2×10^6 cells were 13.00 ± 1.80 and 14.0 ± 4.47 days respectively.

Effect of intradermal inoculation of EL4 lymphoma cells in C57BL/6J mice

The survival of groups of C57BL/6J mice intradermally inoculated with 10^5 (survival of 16.20 ± 1.64) or 10^3 (survival 22.6 ± 0.89 days) EL4 cells/mouse was not different from the survival of groups of mice inoculated with 10^5 or 10^3 EL4 cells i.p. (survival 15.6 ± 1.81, 20.0 ± 1.0 days respectively). The experiments were repeated several times, with comparable results.

'Immunogenicity' and growth of EL4 cells pretreated in vitro *with iodoacetate in PBS for* 1 hr *at* 37°C

Table 2 shows the results of inoculation of 10^7 EL4 cells pretreated with various concentrations of iodoacetate *in vitro*. Pretreatment of EL4 cells even with 0.01 M iodoacetate did not prevent tumour development in the tumour inoculated mice, though the appearance of tumour in these mice was considerably delayed. There was no tumour in comparison groups of mice inoculated with normal syngeneic spleen cells pretreated *in vitro* with 0.005 M or 0.01 M iodoacetate.

Table 2. Survival of C57BL/6J mice inoculated i.p. with 10^7 EL4 cells exposed to various concentrations of oidoacetate in PBS for 1 hr *at* 37°C

Inoculation of 10^7 EL4 cells pretreated with:	Survival (days ± S.D.)
Nil	12.4 ± 0.89
0.001 M Iodoacetate	17.0 ± 0
0.001 M Iodoacetate	32.8 ± 1.30
0.005 M Iodoacetate	39.2 ± 2.05
0.01 M Iodoacetate	94.4 ± 2.97

Immunoprophylaxis with Con A-modified tumour cells

Every member of groups of fifteen mice was injected i.p. with 5×10^5 of either X-irradiated tumour cells or X-irradiated tumour cells coated with Con A, following the method of Martin and Wunderlich [4]. After 4 weeks each mouse received i.p. 5×10^7 tumour, cells prepared as in the first injection. However, consistant with our previous report [7] and that of Johnson *et al.* [10], i.p. inoculation of 10^6 EL4 cells exposed to 1500 rad in C57BL/6J or C57BL/Icrf mice, i.e. a substrain derived from Gorer's colony of C57BL/6 mice in which the EL4 lymphoma originated [1], did not prevent the development of tumour in all tumour inoculated mice irrespective of whether the tumour cells were coated with Con A. Complete tumour suppression could be obtained after exposure of EL4 cells to 2000 rad which was, therefore, the irradiation dose used in this experiment. None of the C57BL mice challenged with 10^4 EL4 cells 10 days after the completion of immunization survived longer than the control group of C57BL mice immunized with irradiated EL4 cells not coated with Con A (survival 18.4 ± 3.5 and 19.2 ± 2.1 days respectively).

Since Martin and Wunderlich, in their experiments with induction of immunity with Con A coated EL4 cells used C57BL/6N mice, i.e. another substrain of C57BL/6 mice maintained for long in the National Institute of Health, U.S.A. [8, 18], the emergence of histoincompatibility between the long transplanted EL4 lymphoma and C57BL/6N mice was thought to be a possibility explaining the complete suppression in this substrain of EL4 cells exposed to 1500 rad and the induction of im

munity by injections of Con A coated EL4 cells [4, 18]. We, therefore, investigated the effectiveness of Con A coated tumour cells in inducing tumour resistance in an allogeneic model, i.e. the EAC in BALB/c mice.

BALB/c mice immunized with Con A modified EAC cells died earlier (i.e. within 10–16 days of challenge) compared to the survival of BALB/c mice immunized with irradiated tumour cells alone (survival 23–31 days). Prior sensitization of groups of C57BL and BALB/c mice to Con A by a method described by Wong *et al.* [19] did not prolong the survival of these mice when challenged as appropriate with Con A coated 10^4 viable EL4 or 2.5×10^6 viable EAC cells.

Immunoprophylaxis against EL4 lymphoma with irradiated tumour cells

Immunoprophylaxis could be consistently induced in C57BL/6J mice using irradiated EL4 cells provided that the number of irradiated EL4 cells per injection and the number of immunizing injections were adequate. Injections containing 10^6, 10^7 or 10^8 irradiated EL4 cells (but not comparable numbers of irradiated syngeneic nucleated spleen cells) given i.p. once a week at least for 3 consecutive weeks, consistently protected varying proportions of mice when they were challenged i.p. with either 10^3 or 10^4 viable EL4 cells/mouse 1 week after the completion of immunization. No such protection could be observed when the number of irradiated EL4 cells per injection was less than 3. The protection increased with increasing numbers of injections and increasing numbers of irradiated EL4 cells per injection. However, immunization with 10^8 irradiated tumour cells (or 10^8 irradiated nucleated spleen cells) i.p. caused ascites and an occasional death due to peritonitis. EL4 specific antibody could be detected in the serum and ascites fluid of the C57BL mice immunized with 10^8 irradiated EL4 cells/mouse but not in mice immunized with comparable numbers of irradiated spleen cells. These anti-EL4 sera were not cytotoxic to EL4 cells *in vitro* and they neither inhibited nor enhanced EL4 lymphoma when injected into tumour-bearing mice [9].

Figure 1 shows that i.p. immunization with 10^7 irradiated EL4 cells/mouse offered better protection against challenge inocula containing 10^3 EL4 cells/mouse than inocula containing 10^4 EL4 cells/mouse, and, also that four i.p. injections of 10^7 irradiated EL4 cells were more protective than four s.c. injections or three i.p. injections followed by one intrasplenic injection of 10^7 irradiated EL4 cells.

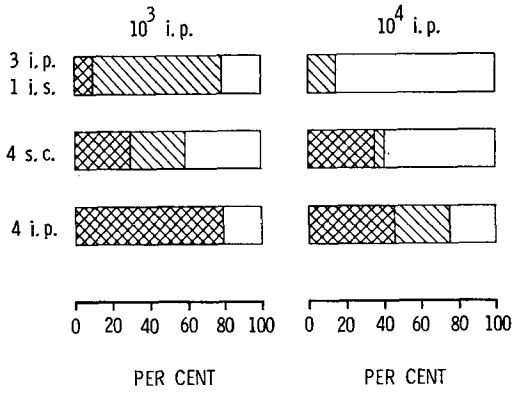

Fig. 1. *Survival of C57BL mice immunized with injections of* 10^7 *X-irradiated EL4 cells/mouse once a week for* 4 *weeks and challenged* 1 *week after the last injection with either* 10^3 *or* 10^4 *viable EL4 cells i.p. Empty space represents per cent of mice dead of tumour within* 30 *days of challenge. Diagonally hatched space represents per cent of mice dead of tumour from days* 31 *to* 200 *post tumour challenge. Cross-hatched space represents per cent of mice surviving tumour-free for* 200 *days.*

The immune C57BL/6J mice that survived a challenge inoculum of 10^4 EL4 cells/mouse were resistant 60 days after the first challenge to further i.p. challenges with 10^5 and 10^6 EL4 cells, but not to a challenge i.p. with 10^7 EL4 cells. However, these immune mice (i.e. those surviving tumour free 60 days after being challenged with 10^4 EL4 cells) had a mean survival of 22.2 ± 2.7 days when rechallenged with 10^4 syngeneic B16 melanoma cells compared to the survival of 22.4 ± 1.9 days of previously untreated C57BL/6J mice inoculated with 10^4 B16 melanoma cells.

Tumour resistance in immunized C57BL/6J mice (not challenged with viable EL4 cells) declined rapidly and was undetectable 16 weeks after immunization as assessed by their survival after i.p. challenge with 10^3 EL4 cells (Table 3). Immunoprophylaxis against EL4 lymphoma with irradiated tumour cells and/or BCG.

Figure 2 shows the survival of C57BL/6J mice challenged i.p. with 10^4 tumour cells after immunoprophylaxis with irradiated EL4 cells and/or BCG. In another experiment the results of different methods of immunoprophylaxis in C57BL/6J mice against the syngeneic EL4 lymphoma was compared with the results of identical methods of immunoprophylaxis against the allogeneic EAC in BALB/ mice (Fig. 3). In immunization protocols 3 and 8, BCG and irradiated tumour cells were incubated together for 10 min at 37°C immediately before injection. The s.c. injections during immunization protocols 5–10 were given at separate sites along the dorsal midline starting

Table 3. Survival of C57BL/6J mice challenged i.p. with 10^3 viable EL4 cells 1, 4, 8 and 12 weeks after completion of immunization with irradiated EL4 cells

Interval between immunization* challenge† (week)	Survivor > 200 days tumour free	Died of tumour between 30 and 200 days post challenge‡	Died of tumour within 30 days (postchallenge)
1	17/21	4/16	4/21
4	6/16	5/14	6/16
8	2/14	5/13	8/13
16	0/14	0/14	14/14§ (All died of ascites tumour between 14 and 18 days after challenge.)

*Immunized with 10^7 irradiated EL4 cells i.p. once a week for 4 weeks.
†Challenged with 10^3 viable EL4 cells per mouse.
‡The majority of the mice in this group did not develop ascites. Autopsy of these mice revealed large spleen and widespread infiltration of internal organs with EL4 lymphoma cells.
§A comparison group of previously untreated C57BL mice challenged with 10^3 EL4 cell died between 17 and 22 days after challenge.

from the caudal end on week 1 and finishing over the neck on week 4. Immunoprophylaxis with BCG alone administered either i.p. (protocol 1) or s.c. (protocol 6) did not protect C57BL mice against challenge with EL4 cells, but both these protocols of immunization offered varying degrees of protection to BALB/c mice against the allogeneic EAC. However, in terms of tumour free survival for more than 200 days in C57BL/6J mice, the protection resulting from i.p. immunization with irradiated tumour cells alone (protocol 2) was increased ($P<0.01$) when the mice were simultaneously immunized with BCG s.c. (protocol 5). As immunization with i.p. injections of only irradiated EAC cells offered complete protection to the BALB/c mice (protocol 2) any additive effect of BCG injections in protocols 3, 4 and 5 could not be elucidated. BCG given i.p. lowered the protection (i.e. tumour free survival > 200 days) produced by s.c. injection of irradiated EL4 cells alone (protocols 7 and 10) in C57BL mice ($P<0.01$), but did not significantly alter the protection offered by s.c. injections of irradiated EAC cells in BALB/c mice. Injections of BCG s.c. at the opposite side (protocol 9) did not increase the protection (i.e. tumour free survival > 200 days) resulting from s.c. injections of irradiated EL4 cells or EAC cells alone (protocol 7). Incubation of irradiated EL4 or EAC cells with BCG prior to subcutaneous injection appears to lower the protection provided by immunization with irradiated EL4 or EAC cells alone (protocol 8).

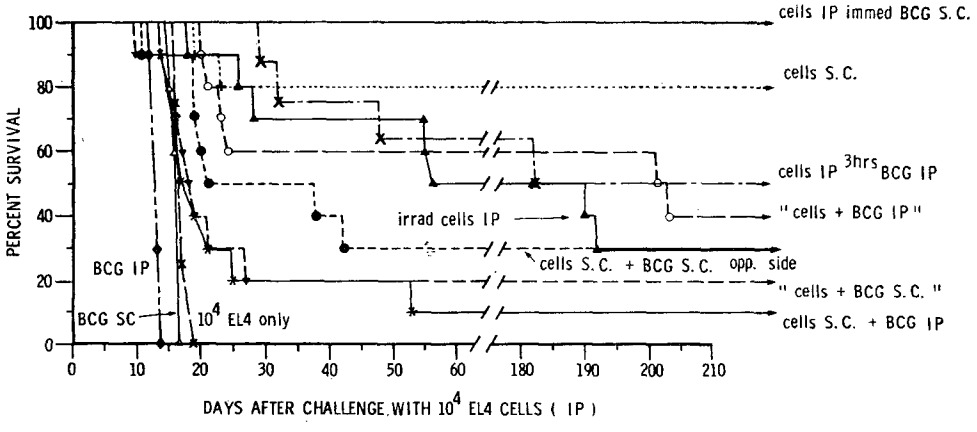

Fig. 2. Survival of C57BL/6J mice challenged with 10^4 EL4 lymphoma cells/mouse 1 week after immunization with injections of 10^7 irradiated tumour cells and/or 10^7 viable BCG once weekly for 4 weeks. 'Cells + BCG' i.p. represents groups of mice inoculated with irradiated EL4 cells incubated with BCG before injection.

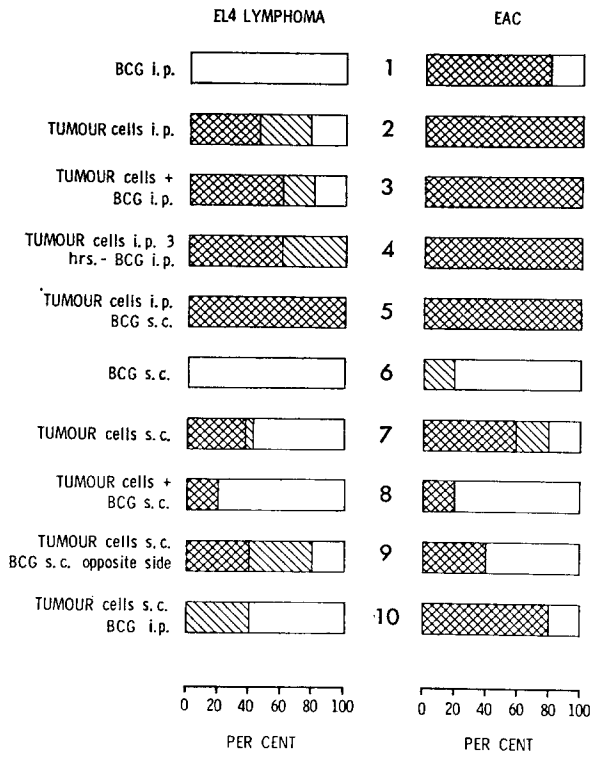

Fig. 3. Comparison of the survival of C57BL and BALB/c mice challenged respectively with 10^4 *EL4 lymphoma and* 2.5×10^6 *EAC cells per mouse 1 week after immunization with injections of* 10^7 *irradiated tumour cells and/or* 10^7 *viable BCG once a week for 4 weeks as explained in the left hand column. For convenience of reference each protocol of immunization has been assigned a number presented in the third column.*

Immunotherapy of EL4 lymphoma with irradiated tumour cells only or irradiated tumour cells and BCG

After i.p. inoculation of 10^2–10^6 viable EL4 cells/mouse, three i.p. injections of 10^8 irradiated EL4 cells were given 2, 48 and 168 hr later. Nine of 10 mice inoculated with 10^2 cells and 1 of 10 mice from groups inoculated with 10^3 and 10^4 EL4 cells/mouse survived tumour-free for 300 days. None of the mice given 10^5 or 10^6 cells before immunotherapy or treated with 3 i.p. injections of irradiated syngeneic nucleated spleen cells survived longer than did the untreated controls. Injection of irradiated tumour cells i.p. followed by BCG s.c., i.e. protocol 5 (Fig. 3), the most potent protocol for immunoprophylaxis, was also used in a modified form for immunotherapy. Two hours after i.p. inoculation of 10^4, 10^5 or 10^6 EL4 cells, each C57BL/6J mouse received injections of 10^8 irradiated EL4 cells i.p. and 10^6 BCG cells s.c. The injections of irradiated EL4 cells and BCG were repeated on days 3 and 7 after tumour inoculation. The survival times of the treated mice receiving 10^5 and 10^6 EL4 cells (14.20 ± 3.19 and 11.25 ± 0.5 days, respectively) were not different from those of untreated controls (15.20 ± 2.28 and 11.40 ± 2.60 days, respectively). However, immunotherapy with BCG and irradiated tumour cells resulted in the survival of two out of twenty mice given 10^4 EL4 cells i.p.

Immunotherapy of EL4 lymphoma with 'adoptive' transfer of syngeneic immune splenic and peritoneal exudate cells

Spleen and peritoneal cells from tumour-free immune C57BL/6J mice 60 days after challenge with 10^3 viable EL4 cells inhibited EL4 cells. This was established by the longer survival i.e. (24.4 ± 3.64 days for splenic cells and 27.6 ± 4.51 days for peritoneal cells) of mice inoculated i.p. with 10^6 EL4 cells mixed with either 10^7 immune nucleated splenic or peritoneal cells compared to the survival of groups of C57BL/6J mice inoculated with 10^6 EL4 cells mixed with splenic or peritoneal cells from previously untreated mice (survival 14.4 ± 1.05 days and 14.4 ± 1.34 days respectively). For immunotherapy, C57BL/6J mice were first given 10^4 viable EL4 cells i.p. and were then treated 1 or 2 hr later with 10^5 immune spleen or peritoneal cells. Survival was slightly prolonged (22 days) in 1 of 10 mice inoculated i.p. with immune spleen cells and 2 of 10 mice (23 and 24 days) given immune peritoneal cells 1 hr after tumour inoculation. The survival times of all other mice in these groups did not differ from the survival of 10 mice inoculated i.p. with 10^4 EL4 cells only (16–19 days).

Active Immunotherapy of EL4 lymphoma with neuraminidase treated tumour cells

Groups of C57BL mice were treated 2 hr after receiving 10^4 EL4 cells/mouse with i.p. injections of either 10^7 EL4 cells exposed *in vitro* to both VCN and mitomycin, mitomycin only or Ml99 only. The mice injected i.p. with VCN and mitomycin treated EL4 cells (survival 15.2 ± 0.44 days) or only mitomycin treated (survival 17.0 ± 1.87 days) or only VCN treated tumour cells (survival 17.2 ± 1.92 days) did not survive longer than the control group of mice which after receiving 10^4 EL4 cells/mouse were injected i.p. with PBS only (survival 17.2 ± 1.92 days). The group of mice, which after receiving 10^4 EL4 cells i.p. were injected with 10^7 EL4 cells exposed to Ml99 only died earlier (survival 11.0 ± 0 days).

Passive immunotherapy of EL4 lymphoma

While examing the biological properties of more than fifty batches rabbit and goat anti-

EL4 sera, 3 batches of rabbit anti-EL4 sera were found to consistently inhibit tumour growth in C57BL mice preinoculated with EL4 cells even though these anti-sera did not render EL4 cells permeable to trypan blue after exposure *in vitro* to these ATG preparations and complement. Table 4 shows the survival of groups of C57BL/6J mice which were tested with 5 injections of 2 mg ATG/mouse/day, either 2, 24 or 120 hr after i.p. inoculation of 10^3, 10^4, 10^5 ATG preparations (Table 4). However, when treatment was started at 120 hr after i.p. inoculation of 10^3 or 10^4 cells/mouse, daily injections of 2 mg ATG (TI)/mouse for 5 days completely suppressed the EL4 lymphoma in a proportion of these mice. Comparison groups of EL4 inoculated C57BL mice treated with 0.1 mg of chlorambucil or 2 mg ATG (NTI) per mouse daily for 5 or 9 days died between 18 and 22 days after tumour inoculation [5, 15].

Table 4. Survival of C57BL/6J mice treated either with tumour inhibitory anti-tumour globulin (ATG) (A) or non-tumour inhibitory ATG bound to chlorambucil (B) or ^{131}I (C) 2, 24, 72 and 120 hr after i.p. inoculation of various numbers of EL4 cells. Each group contained at least 10 mice

No. of EL4 cells inoculated (per mouse) i.p.	Interval—(hr) between tumour inoculation and beginning of treatment and per cent survival after the 3 modalities of treatment, i.e. A, B and C*			
	2	24	72	120
10^3	A—80 B—100 C—100 (400μCi)	A—80 B—100 C—100 (400μCi)	A—80 B—80	A—80 B—0 (P.S.)†
10^4	A—40 B—100 C—100 (540μCi)	A—60 B—90 C—80 (500μCi)	A—50 B—60	A—50 B—0 (P.S.)†
10^5	A—20 B—100			
10^6	A—0 B—60			
10^7	A—0 B—0			

*A—Treated with tumour inhibitory anti-EL4 globulin (2 mg daily for 5 days).
B—Treated with nontumour-inhibitory anti-EL4 globulin bound to chlorambucil (2 mg globulin + 0.1 mg chlorambucil daily for 5 days).
C—Treated with nontumour inhibitory anti-EL4 globulin bound to ^{131}I (approximate activity of ^{131}I in parenthesis).
All injections were intraperitoneal.
†Prolonged survival only, no cure.

or 10^6 EL4 cells/mouse. Eighty per cent of the mice inoculated with 10^3 EL4 cells/mouse and approx 50% of the mice inoculated with 10^4 EL4 cells/mouse survived tumour-free irrespective of the interval between tumour inoculation and treatment.

Immunochemotherapy of EL4 lymphoma with ATG noncovalently bound to chlorambucil

When administered within 72 hr of tumour inoculation, chlorambucil noncovalently bound to a xenogeneic ATG (non tumour inhibitory, NTI) was a better or at least equally effective tumour inhibitor as the tumour inhibitory (TI)

Immuno-radiotherapy of EL4 lymphoma

Xenogeneic ATG (not tumour inhibitory, NTI) when bound to ^{131}I could suppress the growth of EL4 lymphoma in mice preinoculated with 10^4 cells provided that adequate amounts of radio-iodinated ATG was administered within 72 hr of tumour inoculation (Table 4). For tumour inhibition in mice preinoculated with 10^4 EL4 cells/mouse longer than 72 hr it was necessary to administer more than 1 mCi of ^{131}I/mouse, which causes severe bone marrow depression and diarrhaea in these mice. The details and survival of appropriate comparison groups of mice have been described elsewhere [7, 14].

DISCUSSION

The localization of large numbers of i.v. inoculated EL4 cells into the spleen and lymph nodes of C57BL/6J mice is similar to the lymphoid tissue specific localization of i.v. inoculated lymphoma cells in AKR mice [20, 21]. The decreased volume of ascites tumour and increased spleen weight in the C57BL/6J mice inoculated i.p. with $\leqslant 10^5$ EL4 cells compared to the mice receiving $\geqslant 10^6$ EL4 cells i.p. might indicate either localization of EL4 cells in the spleen in preference to the peritoneal cavity in these mice or the ability of these mice to offer effective resistance only against inocula containing $\leqslant 10^5$ EL4 cells.

Increased splenic cellularity and organ weight has been postulated to be associated with tumour resistance in mice bearing syngeneic tumours [22] including lymphomas [23]. It is interesting to note that splenic enlargement, decreased volume (or absence) of ascites and disseminated tumour were also seen at autopsy in those C57BL/6J mice which after immunotherapy or immunoprophylaxis against i.p. inoculated EL4 lymphoma survived longer than tumour inoculated untreated control mice.

Contrary to the report of Rubin [8] that viable EL4 cells after intradermal injection into C57BL/6J mice do not proliferate and induce immunity against further challenges with EL4 the results presented here demonstrate that inoculation of as few as 10^3 EL4 cells into C57BL/6J mice leads to the death with widely disseminated tumour in all tumour inoculated mice irrespective of i.v., i.p., s.c. or intradermal routes of tumour inoculation. This discrepancy might be due to minor degrees of histoincompatibility between the EL4 lymphoma and the C57BL/6N mice, the evidence for which has already been discussed.

In our laboratory, intraperitoneal injection of blood into C57BL/6J mice from mice preinoculated i.p. 24 hr ago with as few as 10^5 EL4 cells/mouse led to the development of EL4 lymphoma in the recipient mice, thus demonstrating the presence of EL4 tumour cells in the circulation of C57BL/6J mice 24 hr after i.p. inoculation of 10^5 EL4 cells/mouse. Further, histological examination of internal organs of C57BL/6J mice inoculated i.p. with 10^6 EL4 cells/mouse had revealed small foci of tumour cells in the lungs 72 hr after tumour inoculation and also definite invasion of the liver and kidney 120 hr after tumour inoculation [7, 15].

Using the 'Pondville subline of C57BL mice', Apffel and Arnason [2] have reported the induction of immunity against EL4 lymphoma after injections of EL4 cells 'inactivated' with 10^{-2}, 10^{-3} and 10^{-4}M iodoacetate. They also reported in this subline of C57BL mice the complete suppression of EL4 lymphoma followed by the development of tumour immunity after serial peritoneal tapping beginning 7–14 days after i.p. inoculation of 2.5×10^6 EL4 cells [3].

However, the suppression of EL4 lymphoma after serial peritoneal tapping [3] and after immunoprophylaxis with iodoacetate treated cells can be explained on the basis of genetic heterogeneity of the C57BL mice used, i.e. a tumour indigenous in the 'C57BL/6 Pondville mice' was rejected by another substrain of C57BL/6 mice used by these investigators [2]. Consistent with our observation, iodoacetate treated tumour cells have been reported to be not very effective in tumour immunotherapy [24] and VCN and mitomycin treated tumour cells are therapeutically effective mostly in tumour models with relatively long survival of tumour inoculated mice [14] compared to the survival of EL4 inoculated C57BL/6J mice.

Studies on immunoprophylaxis and immunotherapy using tumours of long transplantation history like the EL4 lymphoma are complicated by the possibility of viral contamination and the emergence of histoincompatibility between the various tumour sublines and the substrains of mice evolving from the strain of origin of the primary tumour. Our results confirm the effectiveness of immunoprophylaxis with BCG and/or irradiated tumour cells in allogeneic tumours like the EAC [11] or the S91 melanoma in BALB/c mice [25]. On the other hand, immunoprophylaxis with BCG and/or irradiated tumour cells failed in our laboratory to inhibit in AKR mice the syngeneic L2 lymphoma which has a much shorter transplantation history [9]. It is, therefore, not certain that the immunity induced in C57BL/6J mice with injections of irradiated EL4 cells by us and others [9, 10] is truly tumour specific.

The superiority of tumour inhibition by ATG linked cytotoxic agents over tumour inhibition by ATG or cytotoxic agents by themselves is now well established [7, 15, 26–30]. However, although the therapy of malignant tumours with xenogeneic and allogeneic antisera has been generally disappointing [31], more recently there have been reports on the prolongation of survival by a few days, but not 'cell cure' of EL4 bearing C57BL mice [32] and rats bearing a syngeneic lymphoma [33] after injections of appropriate rabbit antitumour sera. The ATG preparations obtained by us appear to be more potent as cell cure could be obtained

in a proportion of mice preinoculated with 10^5 EL4 cells (i.e. 10^4 times more than the minimum number of EL4 cells necessary for 100% tumour take). The complete tumour suppression in a proportion of mice, even when passive immunotherapy was instituted 120 hr after i.p. inoculation with EL4 cells, is especially interesting because there is definite evidence of tumour dissemination 120 hr after i.p. inoculation of EL4 cells and even chlorambucil bound ATG only prolonged the survival and failed to produce any 'cell cure' when treatment was initiated 120 hr after tumour inoculation. We have so far failed to detect tumour inhibition by several goat anti-mouse lymphomas including goat anti-EL4 sera. Further, more than 50 different batches of rabbit and goat antisera against the L2 lymphoma have consistently failed to cause any tumour inhibition. This lack of universality of tumour inhibition by xenogeneic antisera at present limits the usefulness of passive immunotherapy of cancer.

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Water Soluble Derivatives and Bifunctional Analogs of the Anticancer Agent 1, 3-bis(2-chloroethyl)-1-nitrosourea (BCNU)

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Abstract—*Three water soluble and 6 bifunctional newly synthesized analogs of 1,3-bis(2-chloroethyl)-1-nitrosourea* (BCNU) *were screened for anticancer activity against subcutaneously and intracerebrally inoculated Walker carcinosarcoma 256 and 7,12-dimethylbenz(a)anthracene* (DMBA) *induced mammary cancer of the rat. The water soluble 1-(2-hydroxyethyl)-3-(2-chloroethyl)-3-nitrosourea showed a higher activity than BCNU in s.c. inoculated Walker carcinosarcoma 256 in several experiments. Treatment of i.c. inoculated Walker carcinosarcoma 256 with the bifunctional analogs and 1-(2-hydroxyethyl)-3-(2-chloroethyl)-3-nitrosourea resulted in a marked increase in lifespan over untreated rats. However, statistically no differences were found in comparison to the treatment with BCNU. Against the DMBA induced mammary cancer of the rat, only 2 new analogs were examined; they produced a smaller tumor weight inhibition than BCNU. Further investigations are under work to examine the activity of the new compounds against chemically induced brain tumors.*

INTRODUCTION

THE NITROSOUREAS BCNU, 1-2(chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and methyl-CCNU are active in a number of experimental and human tumours. They produce cures in L 1210 leukemia and L 5222 leukemia after intraperitoneal or intracerebral inoculation [1–3] and also cause a marked inhibition of tumor weight or cures in the solid tumor systems B 16 melanoma and Lewis lung carcinoma [4]. In human tumors they have a broad spectrum of activity. BCNU, CCNU and MeCCNU are effective in advanced brain tumors, Hodgkin's disease, non-Hodgkin's lymphomas and melanomas [4–6]. Furthermore BCNU shows some activity against breast cancer [4], and CCNU against lung cancer [6]. In combination therapy they have not yet been investigated adequately [7]. The nitrosoureas are potent alkylators of nucleic acids [8–10]. They are cell cycle nonspecific in their mode of action and also active in slowly proliferating tumors.

BCNU, CCNU and MeCCNU have qualitatively similar toxic effects. Clinically, the main toxic effects observed are acute nausea and vomiting and delayed, dose-limiting myelosuppression [7].

The bifunctional nitrosoureas described here were synthesized to study the influence of bifunctional carbamoylating activity on chemotherapeutic effectiveness [11]. Water soluble compounds were prepared following a suggestion by Hansch *et al.* [12] that the synthesis of more hydrophilic nitrosoureas might uncover less toxic and chemotherapeutically more potent compounds [12].

MATERIAL AND METHODS

Animals. All investigations were carried out with Sprague–Dawley rats kept under conventional conditions. They received Altromin® pellets and water *ad lib.*

Toxicological investigations. For determination of the LD_{50}, at least 4 logarithmically spaced doses were given to 5 male and 5 female rats (3–5 month old) respectively (total number of rats at least 40 for each compound tested). The observation period for the determination of the LD_{50} was 60 days; values were calculated

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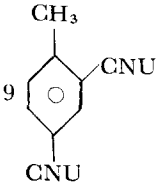
according to the method described by Spearmen and Kärber [13]. All rats were dissected, and the organs with pathological alterations were examined histologically. Early toxicities within 14 day observation period were also given (Table 1).

induced by 7,12-dimethyl-benz(a)anthracene (DMBA). Female Sprague–Dawley rats (50–55 days old) received 3 intravenous injections of DMBA in a total dose of 4 mg per rat within 1 week. The rats were checked weekly for palpable mammary tumors. Estimates of tumor

Table 1. Early toxicity (observation period: 14 days) of 1,1′-polymethylene-bis-[3-(2-chloroethyl)]3-nitrosoureas, 1-(2-chloroethyl)3-(ω-hydroxyalkyl)1-nitrosoureas and 1,1′-(4-methyl-m-phenylene)bis-nitrosourea in rats*

Compound	Solution in % ethanol/ cremophor®: 0.9% saline	Application	50% lethality (mg/kg)	50% lethality 95% confidence interval	10% lethality (mg/kg)
BCNU	5/5 : 90	i.p.	32	29–34	27
CNU-$(CH_2)n$-CNU					
1 $n = 2$	25/25 : 50	i.p.	58	51–64	44
2 $n = 3$	15/15 : 70	i.p.	45	43–47	40
3 $n = 4$	20/20 : 60	i.p.	46	41–52	36
4 $n = 5$	20/20 : 60	i.p.	45	42–48	38
5 $n = 6$	20/20 : 60	i.p.	50	48–52	46
CNU-$(CH_2)n$-OH					
6 $n = 2$	0/0 : 100	i.p.	32	31–33	26
7 $n = 3$	0/0 : 100	i.p.	31	29–34	24
8 $n = 4$	0/0 : 100	i.p.	32	30–35	25
9 	25/25 : 50	i.v.	20	18–23	16

CNU = $Cl-CH_2-CH_2-N(R)-C(=O)-N=N=O$. i.p. = intraperitoneal, i.v. = intravenous. Each dose was tested in 10 rats. *LD_{50} values including late toxicity see text.

Tumors. Male Sprague–Dawley rats (5–7 weeks old) were inoculated subcutaneously with 4×10^6 Walker ascites cells on day 0. Treatment started on day 4, when the tumor had reached approx 0.8 g. On day 8, all rats were killed with ether and the tumors were excised and weighed.

Intracerebral inoculation of Walker 256 ascites cells was carried out according to the method described by Valzelli [14]. A needle (No. 20) with a glass sleeve limiting its penetration to 11 mm was inserted into the petrotympanic-fissure. The rats were anesthesized with ether. Ascites cells (2×10^4 in 25 μl) were implanted into the thalamic area. Pathological examination of randomly selected rats showed that the tumor was growing in a solid form practically exclusively in the target area of the brain (Fig. 1); in rare cases, some tumor cells could be detected additionally within the meninges. Tumor take was 100%.

The autochthonous mammary cancer was size were made by palpation and comparison with plastilin models which had been copied from representative original tumors [15].

The tumors developed multicentrically along the milk-groin. The total tumor mass per rat which was obtained by addition of estimated weights of individual tumors, was taken for evaluation. The first palpable tumors appeared 3 weeks after the application of DMBA. The median induction period until beginning of treatment was 63 days ranging from 42 to 84 days.

Tumors with a longer induction period were not used for chemotherapeutic experiments. The antitumor activity was expressed as tumor weight inhibition,

$$\left[\frac{\text{control-treated}}{\text{control}} \times 100\right],$$

and mean number of tumors per rat. Mortality and changes of body weight were taken as a

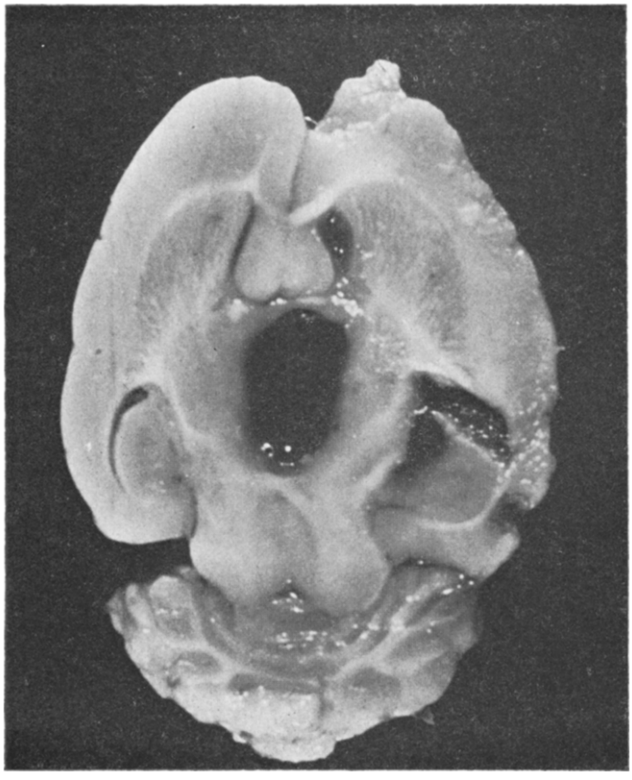


Fig. 1. *Intracerebral growth of Walker* 256. *Untreated rats died* 6 days *after intracerebral inoculation of* 2×10^4 *cells.*

measure for toxic effects. At the beginning of chemotherapy the mean total tumor mass was 3.6 ± 0.6 g and the mean number of tumors was 4.0 ± 1.3 per rat. Each rat had at least one tumor exceeding a weight of 1 g. The rats were randomly assigned into different groups of treatment. All rats were killed 42 days after the beginning of chemotherapy and the tumors were excised and weighed. The tumors from the 18 control rats were examined histologically. Out of 131 tumors, 124 were classified as carcinomas, 5 as preneoplasms, and only 2 as adenomas.

Compounds

Preparation of the compounds and chemical properties have been described elsewhere [11]. Nitrosoureas, not soluble in water, were first dissolved in a mixture of absolute ethanol and Cremophor®-EL* and then made to volume with 0.9% saline solution (Table 1). All substances were given intraperitoneally, except for compound 9 which was given intravenously.

Statistics

Tumor mass or survival time were statistically evaluated by the Kruskal–Wallis test, followed by multiple comparisons [16]. For the evaluation of BCNU against compound 6 the Wilcoxon rank sum test was used (Table 3).

RESULTS

Toxicological investigation

All 9 nitrosoureas showed qualitatively similar toxic effects. Bleedings of the stomach and intestines, lung oedema and a depression of the bone-marrow and lymphoid tissue were observed in animals which died 3–14 days after a single i.p. injection. A marked loss of body weight and moderate leucopenia was observed 40–60 days after the substances had been given. Rats which died of late toxicity showed pronounced liver necrosis and bleedings of the stomach and intestines. Liver-necrosis was not observed after i.v. injection.

The LD_{50} values were determined after an observation period of 60 days to take care of late toxicity of the nitrosoureas. The hydroxylated substances 6–8 and BCNU all had LD_{50} values of 25 mg/kg; LD_{50} values for the bifunctional analogs 1–5 were around 35 mg/kg with a range of 32–40 mg/kg. The aromatic compound 9 was more toxic with a LD_{50} of about 13 mg/kg after i.v. application; (after i.p. application compound 9 produced a peritonite and was therefore given i. v.). LD_{10} values were approximately 20% smaller than LD_{50} values. The early toxicities of the 10 nitrosoureas (observation period of 14 days) are given in Table 1. For all compounds toxicities were similar in both sexes.

Anticancer activity

Tables 2 and 3 summarize the anticancer activity of BCNU analogs in s.c. inoculated Walker 256. BCNU and cyclophosphamide served as reference compounds.

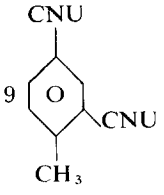
The bifunctional compounds 1–5 showed smaller chemotherapeutic effects than those of BCNU in the 2 schedules tested. One single dose (approx 80% of the LD_{10}) given 4 days after transplantation was more effective than the fractionated schedule given on days 4–7. Single dose application however produced a reduction in body weight, which is a rough parameter of toxicity. The water soluble compounds 6–8 showed striking differences in their activities. Compound 6 was slightly more effective than BCNU in three dose-levels tested; in further experiments, the differences were statistically significant (Table 3). Compounds 7 and 8, however, were practically inactive, although their toxicities were equal to that of 6. The aromatic nitrosourea 9 showed a moderate tumor weight inhibition.

Nitrosoureas used for clinical purposes are known to cross the blood–brain–barrier [17]. Therefore, the newly synthesized nitrosoureas were also tested against i.c. inoculated Walker 256. Control rats died 6–7 days after transplantations of 2×10^4 ascites cells. Treatment with BCNU and the newly synthesized nitrosoureas, listed in Table 4, caused a significant increase in lifespan. However, differences in survival time between BCNU and the new compounds 1–6 were statistically not significant.

In addition to transplantation tumors we used the DMBA-induced mammary cancer of the rat as a tumor model for screening experiments. Table 5 summarizes the results obtained by the treatment with BCNU and the new compounds 3 and 6. In this experiment the new analogs showed an activity which was somewhat smaller than that of BCNU. Schedules with repeated injections were more effective than those with one single injection; they were, however, also more toxic, causing a considerable loss of body weight and increase of mortality.

*A non-ionic solubilizing agent and surfactant based on polyethoxylated ricinus oil (BASF-Ludwigshafen, W. Germany).

Table 2. Anticancer activity of BCNU analogs against subcutaneously inoculated Walker 256

Compound	Dose (mg/kg/day)	Schedule day	Day 8 Tumor weight* (g)	Day 8 Tumor weight inhibition†	Days 4–7 % body weight change
Control			14.9		−2.0
Control			18.6		−8.9
BCNU	16	4	3.2§/18.6	83	−2.7
	6.5	4–7	3.2§/14.9	79	+2.3
	3.3	4–7	9.8 /14.9	34	+5.6
Cyclophosphamide	105	4	1.7§/18.6	91	−2.7
CNU-$(CH_2)n$-CNU					
1 $n = 2$	29	4	3.7§/14.9	75	−3.4
	11.5	4–7	3.9§/14.9	74	−2.8
2 $n = 3$	23	4	4.2§/14.9	72	−1.2
	9	4–7	6.7‡/14.9	55	+5.5
	4.5	4–7	12.7 /14.9	15	+3.6
3 $n = 4$	23	4	3.5§/14.9	77	−3.4
	9	4–7	7.1 /14.9	52	+6.8
	4.5	4–7	12.7 /14.9	15	+5.4
4 $n = 5$	23	4	4.8§/14.9	68	+0.9
	9	4–7	7.1‡/14.9	52	+2.5
	4.5	4–7	14.4 /14.9	3	+5.8
5 $n = 6$	25	4	4.5‡/14.9	70	0.0
	10	4–7	7.0 /14.9	53	+4.7
CNU-$(CH_2)n$-OH					
6 $n = 2$	16	4	2.7§/18.6	85	+2.2
	6.5	4–7	2.6§/14.9	83	−2.5
	3.3	4–7	7.9§/14.9	47	−2.1
7 $n = 3$	6.5	4–7	18.8 /18.6	0	−3.3
8 $n = 4$	6.5	4–7	17.9 /18.6	4	−2.1
9 	10	4	9.7 /14.9	35	−3.6
	4.0	4–7	11.6 /18.6	38	−2.5

Inoculation size 4×10^6 ascites cells. Each dose was tested against 10 Sprague–Dawley rats. Compounds were given i.p.; only compound 9 was injected intravenously. *Mean of experimental over mean of controls for that experiment. † $\frac{\text{control–test}}{\text{control}} \times 100$. ‡$P$ versus control < 0.05. §$P < 0.01$ (Kruskal-Wallis).

Table 3. *Comparison of anticancer activity of BCNU and* 1-(2-*hydroxyethyl*)-3-(2-*chloroethyl*)-3-*nitrosourea* (*Compound* 6) *against subcutaneously inoculated Walker* 256

Compound	Number of rats	Dose* (mg/kg)	Tumor weight day 8 (g) T/C†	*P*-value‡
BCNU	22	16	2.7 ± 1.3/15.4 ± 3.2	$P < 0.01$
Compound 6	22	16	2.0 ± 1.3/15.4 ± 3.2	
BCNU	13	20	2.3 ± 1.1/13.3 ± 2.6	$P < 0.1$
Compound 6	14	20	1.7 ± 0.8/13.3 ± 2.6	
BCNU	14	12.5	4.6 ± 1.9/13.3 ± 2.6	$P < 0.05$
Compound 6	14	12.5	2.9 ± 1.5/13.3 ± 2.6	
BCNU	14	7.8	9.6 ± 4.3/13.3 ± 2.6	$P < 0.1$
Compound 6	14	7.8	8.3 ± 2.9/13.3 ± 2.6	

*Single dose given i.p. on day 4. LD_{10} for both compounds is 20 mg/kg.
†Mean ± standard deviatiation of treated groups in comparison to controls.
‡Wilcoxon rank sum analysis.

Table 4. *Anticancer activity of BCNU analogs against intracerebrally inoculated Walker* 256

Compound	Dose (mg/kg/day)	Schedule (day)	MST* (days) Median	MST* (days) Range	ILS† (%)
Control			6	6–7	
BCNU	16	3	13§	11–18	117
	8	3–6	12.5§	5–16	108
CNU-$(CH_2)n$-CNU					
1 $n = 2$	29	3	13§	6–15	117
	20	3–6	13.5§	12–17	125
2 $n = 3$	23	3	11	4–14	83
	16	3–6	12‡	7–18	100
3 $n = 4$	23	3	11.5	6–14	92
	16	3–6	13§	5–15	117
4 $n = 5$	23	3	12‡	5–14	100
	16	3–6	12.5§	12–16	108
5 $n = 6$	25	3	13§	10–18	117
	20	3–6	13§	5–16	117
6 CNU-$(CH_2)_2$-OH	16	3	12§	10–18	100
	8	3–6	12.5‡	5–15	108

Inoculation size 2×10^4 tumor ascites cells. Each dose was tested against 10 rats. Doses are equivalent to the optimal dose, determined against intramuscular Walker carcinosarcoma 256. Compounds were given intraperitonally. *Median survival time. †Increase in lifespan in % test over control. ‡P vs control < 0.05. §$P < 0.01$ (Kruskal–Wallis test).

Table 5. Anticancer activity of BCNU and 2 analogs against the autochthonous DMBA induced mammary cancer of the rat

Compound	Dose (mg/kg/day)	Schedule day	Mean tumor weight* (g) day 0	21	42	% TWI day 42	Mean tumor No.* day 0	21	42	Mortality day 42/ total day 0	Body weight* change in % day 0–42
Control			13.4	11.8	13.7		4.2	6.7	7.1	0/20	+ 6.5
BCNU	16	1	4.2	8.1	9.3	32	4.0	7.3	7.4	1/9	+ 0.1
	8	1–5	2.9	0.6§	0.4§	97	3.4	2.0	1.5	0/10	− 32.7
	8	(1.4) × 3 weeks	3.0	2.3§	1.0§	93	2.3	3.0	2.9	1/10	− 26.5
6 CNU–CH_2–OH	16	1	3.0	4.0	10.4	24	4.3	5.2	7.4	0/9	+ 2.9
	8	1–5	3.0	1.6§	0.5	96	3.2	3.1	1.9	1/10	− 13.3
	8	(1.4) × 3 weeks	3.7	3.8	2.6	81	4.0	3.4	3.9	3/10	− 7.0
3 CNU–$(CH_2)_2$–CNU	23	1	3.5	5.8	9.7	29	3.2	5.5	7.3	0/10	+ 3.5
	16	1–5	4.4	4.1‡	1.8‡	87	5.0	4.6	3.1	2/9	− 11.3
	16	(1.4) × 3 weeks	4.5	3.3§	1.7§	88	4.7	4.0	3.0	2/10	− 12.2

TWI-Tumor weight inhibition = $\frac{\text{control–test}}{\text{control}} \times 100$. *Calculations were based on survivors only. ‡P vs control < 0.05. §$P < 0.01$ (Kruskal-Wallis test).

DISCUSSION

Screening for anticancer activity of BCNU and 9 newly synthesized analogs in the s.c. Walker 256 revealed one analog which produced a statistically higher tumor inhibition than BCNU. This was compound 6, 1-(2-hydroxyethyl)-3-(2-chloroethyl)-3-nitrosourea which is a water soluble analog of BCNU. The lipophilic compounds 1–5, however, were somewhat less active than BCNU. No relationship could be found between the length of the polymethylene bridges and antitumor or toxic effects of these bifunctional congeners of BCNU. The introduction of bifunctional carbamoylating activity into the nitrosourea molecule therefore does not influence its biological properties significantly.

Treatment of i.c. inoculated Walker 256 with BCNU, the bifunctional lipophilic analogs 1–5 and compound 6 showed no statistical differences in survival time: all compounds displayed equal activities. However, compounds 1–5 were somewhat less active against s.c. implanted Walker 256 than BCNU. The relatively better chemotherapeutic effectiveness of the latter compounds against i.c. inoculated tumor cells might be attributed to an increased ability to cross the blood brain barrier.

Further investigations using slower growing models of brain tumors which better reflect the characteristics of human neoplasm are in progress.

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Proteases During the Growth of Ehrlich Ascites Tumor. III. Effect of *e*-Aminocaproic Acid (EACA) and Heparin*

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Abstract—*The effect of heparin and e-aminocaproic acid (EACA) on the* in vivo *growth of Ehrlich ascites tumors was studied. The drugs were administered intraperitoneally every* 8 hr *for* 8 *consecutive* days *beginning* 16 hr *after transplant of* 10 *million tumor cells. Heparin doses studied ranged from* 50 *to* 800 units/kg *and EACA doses were* 125–2000 mg/kg. *Criteria for therapeutic effect included measurement of total ascites volume (TAV) and total packed cell volume (TPCV) determined* 16 hr *after the last drug injection. Both heparin and EACA exerted a dose-dependent chemotherapeutic effect. Heparin, at the highest dose of* 800 units/kg *significantly reduced TAV by an average of* 46.9% ($P<0.01-<0.005$) *and reduced the TPCV by an average of* 46.1% ($P<0.01-<0.005$). *At the lowest heparin dose of* 50 units/kg *a biphasic inhibitory response was obtained reflected by a decrease in both TAV* (26.1%, $P<0.025-<0.01$) *and TPCV* (26.3%, $P<0.05-<0.005$). *EACA, at the highest dose of* 2000 mg/kg, *inhibited TAV by* 56.3% ($P<0.001$) *and TPCV by* 48.1% ($P<0.001$).

INTRODUCTION

WITHIN recent years, attention once again has focused on the possible relationship between protease activity in malignant tissue and *in vitro* transformed cells and tumor growth and development [1–6]. Moreover, both tumors and transformed cells have been reported to be affected adversely by protease inhibitors studied *in vitro* [3, 7–11] and *in vivo* [4, 12–16], as well as by heparin [17].

Studies in our laboratory have explored the possible role of proteases in certain phases of tumor growth [5, 16, 18]. This investigation was designed to study the potential chemotherapeutic effect of *e*-aminocaproic acid (EACA) and heparin on the *in vivo* growth of the Ehrlich ascites tumor.

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MATERIAL AND METHODS

Tumor transplantation

Ehrlich ascites tumor, provided by Dr. P. Hebborn of our department, was maintained by weekly intraperitoneal transplant in male albino Swiss mice, ranging in weight from 20 to 25 g. Tumor transplant was carried out as described previously [5]. On the 7th or 8th day after maintenance transplant, donor mice were killed by cervical fracture and the ascites fluid drained from the abdominal cavity into sodium citrate, 3.8%, in a ratio of 1 ml/10 ml ascitic fluid. The tumor cell number was estimated by microscopic cell count and the tumor cell viability confirmed with lissamine green [19]. The tumor cell suspension was diluted with physiologic saline to a concentration of 20 million cells per ml and 10 million cells (0.5 ml suspension) were transplanted into the lower quadrant of the peritoneal cavity of experimental recipient mice.

Treatment

Sixteen hours after tumor transplant, therapy was stated every 8 hr for 8 consecutive days. The drugs studied included heparin (Pan-Heparin, Abbott Labs., North Chicago, Ill) at doses of

50, 100, 200, 400 and 800 units/kg, and *e*-aminocaproic acid (EACA; Lederle Labs., Pearl River, N.Y.) at 125, 250, 500, 1000 and 2000 mg/kg doses. A volume of 0.2 ml of the drug or saline (control) solution was injected into the lower abdominal quadrant opposite the implantation site. Every 2 days a contralaterial injection site was used. Twenty mice constituted the saline control groups and 10 mice for each drug treatment group.

Evaluation of therapeutic effect

The ascites tumors were harvested 16 hr after the last injection of drug. The carcass weights of the animals in each group were recorded and compared to the initial pre-treatment weights as an indication of general drug-induced toxic effect. As an index of tumor inhibition [20], the total packed cell volume (TPCV = ascites volume in a mouse × percentage of packed cell measured in an IEC model MB (Boston, Mass.) micro-capillary centrifuge) was determined. Total ascites volume (TAV) also was recorded as an indication of the extent of fluid effusion into the peritoneal cavity. The means of the TAV and TPCV of the drug-treated and control groups were compared (±S.E. of mean) and levels of significance calculated using the *t* test method.

RESULTS

Table 1 summarizes the effect of heparin on the total ascites volume (TAV) and total packed cell volume (TPCV) in 2 individual experiments. While the control means of both the TAV and TPCV varied between the 2 experiments, the relative effects of the respective heparin doses were similar. At the lowest dose of 50 units/kg, heparin reduced the TAV by 21.9% ($P<0.001$) and 30.2% ($P<0.025$) respectively with an average of 26.1%. At that low dose, heparin inhibited tumor growth (TPCV) by 18.2% ($P<0.05$) and 34.3% ($P<0.005$) with an average of 26.3%. This inhibitory effect was exceeded only at the highest heparin dose studied, 800 units/kg, where TAV was decreased by 36.0% ($P<0.005$) and 57.7% ($P<0.001$) and TPCV inhibited by 44.2% ($P<0.005$) and 48.0% ($P<0.01$). Exclusive of the lowest dose of heparin studied, a dose–response relationship obtained relative to the two parameters measured. No significant differences between the initial weight and carcass weight were noted in mice receiving saline and those receiving heparin up to 400 units/kg. The mean weight increase (carcass weight minus initial weight) in the mice treated with 800 units/kg heparin was 0.1 g ± 0.15 while in all other treatment and control groups the mean weight increase ranged from 1.6 to 2.8 g.

The effect of EACA on the TAV and TPCV of the Ehrilich ascites tumor is summarized in Table 2. EACA inhibited the accumulation of the ascites fluid (TAV) in a dose-related manner. At the lowest dose, 125 mg/kg, EACA inhibited the TAV 19.0%. As the dose was increased, less ascites fluid accumulated. The highest dose of EACA studied, 2000 mg/kg, resulted in an inhibition of TAV by 56.3% ($P<0.001$). The 53.2% inhibition at the 1000 mg/kg dose was significant at the 0.001 level. EACA also inhibited the tumor growth at all dose levels. The highest percent of TPCV inhibition was obtained at the 1000 mg (53.5% $P<0.001$) and 2000 mg/kg (48.1%, $P<0.001$) doses. Mean weight increases in mice receiving 1000–2000 mg/kg EACA during the 8-day treatment period was 0.3 ± 0.15 g compared to the mean weight increases of 1.8–2.6 g for the control and other experimental groups.

Table 1. Effect of heparin administered every 8 hr *for* 8 days *on the total ascites volume (TAV) and total packed cell volume (TPCV) during the growth of Ehrlich ascites tumors*

	Total ascites volume (TAV)				Total packed cell volume (TPCV)			
	Experiment No. 1		Experiment No. 2		Experiment No. 1		Experiment No. 2	
Dose Units/kg.	Mean (ml) ±S.E.	Inhibition (%)	Mean (ml) ±S.E.	Inhibition (%)	Mean (ml) ±S.E.	Inhibition (%)	Mean (ml) ±S.E.	Inhibition (%)
Control	3.47 ± 0.21		2.08 ± 0.21	—	1.92 ± 0.11	—	1.02 ± 0.30	—
50	2.71 ± 0.19	21.9	1.45 ± 0.20	30.2	1.57 ± 0.17	18.2	0.67 ± 0.13	34.3
100	3.52 ± 0.19	(+)1.4*	2.00 ± 0.13	3.84	1.96 ± 0.04	(+)2.0*	1.06 ± 0.08	(+)3.7*
200	2.97 ± 0.16	14.4	1.85 ± 0.25	11.2	1.71 ± 0.16	10.9	0.96 ± 0.10	5.8
400	2.71 ± 0.28	21.9	1.68 ± 0.11	19.2	1.40 ± 0.17	27.0	0.85 ± 0.24	16.6
800	2.22 ± 0.19	36.0	0.88 ± 0.17	57.7	1.07 ± 0.01	44.2	0.53 ± 0.40	48.0

*(+) indicates the percent increase in mean TAV and TPCV over control values.

Table 2. Effect of e-aminocaproic acid (EACA) administered every 8 hr *for* 8 days *on the total ascites volume (TAV) and total packed cell volume (TPCV) during the growth of Ehrlich ascites tumors*

Drug (mg/kg)	Total ascites volume (TAV) Mean (ml) (±S.E.)	Inhibition (%)	Total packed cell volume (TPCV) Mean (ml) (±S.E.)	Inhibition (%)
Control	3.57±0.38	—	1.87±0.22	—
125	2.89±0.32	19.0	1.36±0.19	27.3
250	2.85±0.18	20.0	1.61±0.09	13.9
500	2.55±0.28	28.6	1.50±0.18	19.8
1000	1.67±0.19	53.2	0.87±0.14	53.5
2000	1.56±0.17	56.3	0.97±0.14	48.1

DISCUSSION

The results reported here demonstrate that, in appropriate doses, both EACA and heparin significantly inhibit Ehrlich ascites tumor growth. Component levels of both the fibrinolysis [5, 21] and the vasopeptide kinin systems [18, 22], present in the ascites tumor fluid, were reported in dynamic change during various phases of tumor growth, suggesting that these systems are active during the tumor growth process. This suggestion receives further support from the results obtained with EACA, a known inhibitor of plasmin and plasminogen activation. In addition, EACA was found to be a weak inhibitor of plasma and urinary kallikrein [23], and a moderate inhibitor of pancreatic kallikrein [23] and rodent plasma and tumor tissue kallikrein [24]. Thus, the tumor chemotherapeutic effect of EACA may relate to its action on vascular-tumor protease systems possibly active during tumorigenesis [4].

In this study, heparin also was shown to decrease the growth of the Ehrlich ascites tumor. A recent review has summarized previously published reports on the tumor-inhibitory effect of heparin [17]. The actions of heparin are complex and its tumor-inhibitory mechanism not clear. Heparin was found to be a mitotic inhibitor of ascites tumor cells *in vivo* [25], an activity not expressed in the absence of a plasma coagulum [26]. Heparin was found to inhibit trypsin [27], thrombin [28] and, at high concentrations, the kinin-forming activity of plasma and tissue kallikrein [29]. Heparin did inhibit the growth of solid rodent tumors without exerting influence on the tumor vasopeptide kinin protease system [16]. The possible antitumor potential of heparin also has been viewed in terms of its anticoagulant action [17]. The reported *in vitro* inhibition of plasma fibrinolytic activity by heparin [30] may be of importance, if indeed there is a link between fibrinolysis and tumor growth, an area recommended for further study by Reich [2]. The biphasic inhibitory effect of heparin noted in this study may relate to findings that heparin enhances fibrinolysis in low concentrations but exerts an inhibitory effect in higher concentrations [31].

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Changes in Cellular Immunity Following Nephrectomy for Localized and Metastatic Hypernephroma

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Abstract—*A group of sixteen patients with untreated hypernephroma were shown to have significantly reduced PHA induced cytotoxicity and antibody dependent cellular cytotoxicity compared with the same patients* 10–14 days *post nephrectomy, or with normal controls. The T cell and non T (K) cell function improved following nephrectomy and values were reached not significantly different from the normal controls. The influence of tumour burden was also investigated. Six patients had demonstrable distant metastatic disease preoperatively and ten patients were without clinically evident metastases and were apparently "tumour free" following operation. No significant differences were found between these two groups of patients. The known occurrence of "spontaneous" regression in metastatic tumour after palliative nephrectomy raises speculation as to the* in vivo *significance of the increase in cytotoxicity described in this* in vitro *investigation of immune function.*

INTRODUCTION

HYPERNEPHROMA is one malignant neoplasm in which an effective host defence mechanism is clinically manifest. This is illustrated by occasional indolent activity, with slow growth of the primary tumour and the development of metastases after prolonged latent periods [1]. The rare spontaneous regression of these tumours, particularly of pulmonary metastases, is also well documented [2]. The regression of metastases has been particularly evident following ablation of large primary tumours and provides good evidence for host resistance to this neoplasm [3].

Laboratory investigation of leucocyte function using different assay systems has given support for an immune reaction directed towards the renal carcinoma [4–7]. However, little information is available concerning the correlation of these immune responses to the clinical status of the tumor and to the effects of therapy used. One report has described a higher leucocyte reactivity in the blood of patients with early stage as compared with late stage disease [7], but other reports have not confirmed this relationship [4, 5].

The present investigation was undertaken to determine the influence of nephrectomy on lymphoid cell effector function, in localized disease where operation removed all known tumour and in metastatic disease where tumour remained behind after nephrectomy. This investigation would also be interesting in the light of the rare metastatic regressions occurring after nephrectomy for hypernephroma.

The tests chosen were of antibody dependent cellular cytotoxicity (ADCC), phytohaemagglutinin stimulated cytotoxicity (PCC) and direct cellular cytotoxicity (DCC), against a Chang target cell. Different subpopulations of blood lymphocytes are thought to be involved with these effector functions and provide a useful assessment of changes in immune reactivity *in vitro*.

Antibody dependent cellular cytotoxicity is a function of "non-T cells" [8], but the effector cell ("K" cell) is separate from the B cells which are precursors of antibody forming cells [9]. The PHA stimulated cytotoxicity would appear to reside mainly in the T cell subpopulation [10, 11]. The identity of the human effector cell responsible for spontaneous, non-specific cytotoxicity is unclear. It has been reported to bear Fc receptors [12, 13] and is found in the non T cell population [14, 15].

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MATERIAL AND METHODS

Patients

The details of sixteen patients with hypernephroma are summarized in Table 1a. No patient had received previous radiotherapy, chemotherapy or hormone therapy. Ten patients were classed "non-metastatic" as having no distant metastases at time of investigation. These patients were, apparently, tumour free following nephrectomy (Table 1a). The "metastatic" group comprised 6 patients with multiple deposits involving lung, liver and bone (Table 1a). Although these patients had a nephrectomy, no further attempt at reduction of tumour volume, e.g. resection of metastases, was made at this time. Blood transfusions were given post operatively as shown (Table 1a). The mean age of the patients was 55.3 yr (range 33–76). The normal controls were 14 individuals employed in the hospital service and seven relatives of patients (Table 1b). The mean age of the controls was 46.9 yr (range 32–68).

Lymphocyte preparations

The patient blood samples were obtained on the day of operation prior to premedication and at 11–14 days after operation. The blood samples were tested on the same day as collection. Lymphocyte suspensions for cytotoxic assays were prepared from defibrinated peripheral blood after incubation with finely divided iron and sedimentation with a magnet at 37°C in 1% methylcellulose. The lymphocyte-rich supernatant was washed three times in minimal essential medium (MEM) and the concentration adjusted to 3×10^5 cells/ml in MEM supplemented with 10% heat inactivated foetal calf serum, 2mM glutamine, 100 I.U./ml streptomycin, 200 I.U./ml penicillin and $NaHCO_3$ buffer. The lymphoid cells comprised 97% or more of the leucocytes present in these suspensions.

Cytotoxic assay

Chang cells labelled with ^{51}Cr sodium chromate (100–350 mCi/mM, Amersham, Bucks.), were washed and diluted to give a final concentration of 10^4 cells/ml in supplemented MEM. The ^{51}Cr release assay of non-specific cytotoxicity against antibody sensitized Chang target cells [16, 17] and the phytohaemag-

Table 1a. *Effect of nephrectomy on lymphocyte cytotoxicity in non-metastatic patients and metastatic patients*

Patient	Sex	Age	Prenephrectomy Mean corrected % ^{51}Cr release D.C.C.	A.D.C.C.	P.C.C.	Post nephrectomy Mean corrected % ^{51}Cr release D.C.C.	A.D.C.C.	P.C.C.
A.A.	M	39	1.6	11.4	16.0	4.5	14.4	24.6
N.B.	M	57	0	33.3	30.3	13.7	20.0	29.5
J.Y.	M	64	12.7	21.8	16.5	12.2	58.0	30.1
N.L.	F	33	14.8	26.9	22.3	11.7	47.0	26.8
L.R.	M	53	0	9.0	10.1	12.8	38.8	43.2
*H.B.	M	54	1.8	43.8	27.8	5.3	58.0	20.7
G.M.	M	76	23.4	44.2	22.7	6.6	45.2	31.9
F.W.	M	59	29.7	21.6	56.3	43.3	73.2	64.3
G.M.	M	69	9.2	51.1	46.5	2.8	61.5	54.4
S.S.	M	64	13.0	27.1	41.7	29.3	39.4	81.5

*Blood transfusion (maximum 3 units packed cells).

Patient	Sex	Age	Prenephrectomy Mean corrected % ^{51}Cr release D.C.C.	A.D.C.C.	P.C.C.	Post nephrectomy Mean corrected % ^{51}Cr release D.C.C.	A.D.C.C.	P.C.C.	Metastases
†A.B.	M	72	5.3	23.7	14.2	5.7	26.3	47.0	P
M.R.	F	62	15.3	78.6	70.1	35.0	70.1	78.7	P, H
†B.S.	M	37	1.9	30.8	36.6	8.8	35.3	53.0	P
S.M.	F	44	5.6	4.4	36.3	3.5	20.4	32.1	P
K.M.	F	59	5.1	21.0	29.5	28.4	51.6	42.8	O
C.M.	F	42	18.3	66.2	48.2	3.5	55.3	30.5	P, H

†Blood transfusion (maximum 3 units packed cells). P—pulmonary, H—hepatic, O—osseous.

Table 1b.

Test data for normal controls					
			Mean corrected % ^{51}Cr release		
Name	Sex	Age	D.C.C.	A.D.C.C.	P.C.C.
D.C.	M	38	5.0	47.6	71.0
H.B.	M	32	9.4	44.7	67.9
W.Y.	M	39	23.6	35.9	34.7
D.B.	F	39	27.5	52.1	33.0
I.T.	M	49	14.2	47.2	53.1
L.S.	M	54	26.3	51.3	41.4
S.M.	M	60	10.1	42.0	37.5
*A.A.	F	59	9.7	18.4	48.6
M.S.	M	33	20.8	38.6	9.8
G.T.	M	66	15.1	23.8	58.6
*J.B.	M	63	22.3	38.0	44.1
*B.W.	M	51	26.9	40.3	40.6
*P.W.	M	61	19.0	30.8	47.2
C.G.	M	33	16.2	39.3	43.6
A.D.	M	53	21.3	55.0	37.3
S.F.	F	54	13.7	35.1	27.7
*D.D.	M	58	8.0	34.0	45.7
*R.N.	M	68	19.7	45.9	43.1
A.L.	F	47	6.1	39.1	57.1
S.S.	M	42	2.0	46.8	29.2
*R.H.	M	49	15.0	45.2	45.1

*Indicates patient's relatives.

Table 2a. Comparison of median values for non-metastatic and metastatic patient groups

	Prenephrectomy			Pre to post nephrectomy change		
	Non metastatic vs metastatic		*P**	Non metastatic vs metastatic		*P**
DCC % MCR	11:0	5.5	NS	3.2	3.7	NS
ADCC % MCR	27.0	27.3	NS	13.3	3.6	NS
PCC % MCR	25.3	36.5	NS	8.9	11.0	NS

Table 2b. Comparison of medians for prenephrectomy, post nephrectomy and normal controls. (Metastatic and non-metastatic patients combined)

	Prenephrectomy (1)	Post nephrectomy (2)	Normal controls (3)	Differences between 1+2+	1+3*	2+3*
DCC % MCR	7.4	10.3	15.1	NS	NS	NS
ADCC % MCR	27.0	46.1	40.3	$P<0.02$	$P<0.031$	NS
PCC % MCR	29.9	43.0	43.1	$P<0.01$	$P<0.05$	NS

% MCR—Mean corrected % ^{51}Cr release.
*Statistical analysis by Mann–Whitney U Test.
+Statistical analysis by Wilcoxon matched-pairs signed rank test.
NS—not significant $P>0.1$.

glutinin stimulated lymphocytotoxicity against the labelled Chang cell [18, 19] were performed as follows. The cultures were set up in triplicate, containing 1 ml of lymphocytes and 1 ml of Chang cells. The test was divided into three sections. Chang cells were reacted with lymphocytes alone—DCC; with lymphocytes and rabbit anti-Chang serum diluted $1:10^5$—ADCC; and lymphocytes with phytohaemagglutinin, PHA (Wellcome Reagents Ltd.); 3 μg/ml—PCC. Control tubes were set up with each experiment containing Chang cells alone, with antibody or with PHA, but without lymphocytes. These tubes gave the spontaneous ^{51}Cr release. Maximal ^{51}Cr release was obtained by lysis with distilled water. After standing at 37°C in a 5% $C0_2$ incubator for 20 hr the tubes were centrifuged and 0.5 ml supernatant transferred to empty tubes and counted on a gamma counter. The counts/min were printed onto a punch tape which was processed by a digital computer.

The ^{51}Cr percentage release was computed for each tube from

$$\frac{\text{supernatant counts/min} \times 4}{\text{total counts/min (pellet and supernatant)}} \times 100.$$

The maximal release varied between 82–90% and the spontaneous release between 20–34%.

Results were expressed as the mean corrected percentage ^{51}Cr release (M.C.R.) of the triplicates, and obtained from

$$\frac{\text{experimental } ^{51}Cr \text{ percentage release} - \text{spontaneous } ^{51}Cr \text{ percentage release}}{\text{maximal } ^{51}Cr \text{ percentage release} - \text{spontaneous } ^{51}Cr \text{ percentage release}} \times 100.$$

The S.D. between triplicates was ± 3.8.

RESULTS

The test values before nephrectomy of the "non-metastatic" and "metastatic" patient groups were compared. No statistically significant difference was found, although the DCC, median 5.5, was lower whilst the PCC, median 36.5, was higher in the "metastatic group" compared with the "non-metastatic" values of 11.0 and 25.3 respectively (Table 2a).

The changes following nephrectomy were also compared for the two patient groups (Table 2a). Again no significant difference was found between these changes in "non-metastatic" patients and the changes in "metastatic" patients. However, the change in ADCC values was smaller for the "metastatic" patients (median 3.6) than the change for "non-metastatic" patients (median 13.3).

Since the prenephrectomy values for the "metastatic" and "non-metastatic" groups showed no significant difference and there was no significant difference in the changes with nephrectomy between the two patient groups, it was concluded that there was no statistically significant difference between the post nephrectomy "metastatic" and "non-metastatic" patient groups. The values of the "metastatic" and "non-metastatic" groups could, therefore, be combined for the remainder of the analysis.

The prenephrectomy results for all patients were accordingly compared with the post nephrectomy results (Table 2b). The prenephrectomy, ADCC values (median 27.0) and PCC values (median 29.9) were significantly lower ($P<0.02$, $P<0.01$ respectively) than the post nephrectomy values (median ADCC 46.1, median PCC 43.0). The DCC test values were also lower (median 7.4) prenephrectomy, compared with the post nephrectomy values (median 10.3), but this difference was not significant ($P>0.19$). The prenephrectomy values for the ADCC and PCC tests were also significantly less ($P<0.031$ and $P<0.05$ respectively) than the corresponding values for normal controls (Table 2b). The prenephrectomy DCC test value when compared with normals showed a similar trend, but again the difference was not significant ($P>0.1$). No significant difference was found between the post nephrectomy test values and the normal control values.

DISCUSSION

Non-specific cytotoxicity mediated by T cells, (PCC) [10, 11] and non T, K cells, (ADCC) [8, 9] was found to be significantly depressed in patients before nephrectomy, compared with the same patients after nephrectomy or with normal controls. A similar depression was also seen in the DCC test, another non T cell function [14, 15], but this was not statistically significant. Other tests of T cell function, e.g. PHA lymphocyte blastogenesis have shown depressed values in cancer patients [20], but non T cell function has received less attention. The depression in ADCC values of unresected hypernephroma patients is supported by another study using lysis of antibody coated normal lymphocytes by lymphocytes from patients with a variety of tumour types [21].

After nephrectomy, the depression in T cell and non T cell function was abrogated and the

post operative tests were not significantly different from those of the normal controls. The increase in post operative cytotoxicity could not be explained by blood transfusion sensitization as only 3 patients were transfused during the test period and in these 3 the rise in cytotoxicity (particularly ADCC) was modest. Few reports are available demonstrating an increase in nonspecific cytotoxicity following operation, but such an increase has been described in bronchogenic carcinoma [22].

An allied situation concerns the relationship of tumour burden or tumour stage and various *in vitro* assays of immune function. This remains an area of considerable controversy. A more profound decrease in nonspecific lymphocytotoxicity has been noted for late stage compared with early stage disease in bronchogenic carcinoma [22], various cancers [21] and in hypernephroma [7], but only the latter report demonstrated statistically significant differences. Other tests such as PHA blastogenesis using "sub-optimal" PHA concentrations have also demonstrated a depressed responsiveness which increased in severity with the extent of malignant melanoma [23] and several authors have described a diminution in "tumour specific" cellular immunity with increasing dissemination of this tumour [24, 25]. Similar observations have also been made with bladder carcinoma: low stage tumour patients had cytotoxic lymphocytes in almost all cases, but the frequency of positive cases was significantly less when tumour growth was extensive [26]. In contrast to these reports the present study of hypernephroma did not demonstrate a significant difference between the preoperative tests of the "metastatic" and "non-metastatic" patient groups, and no significant difference in the changes of cytotoxicity from pre to post nephrectomy for the two groups. This lack of correlation of *in vitro* cytotoxicity with tumour stage agrees with other studies in hypernephroma [4, 5] and investigations of other tumours [27]. Although in the latter study a reduction in cellular immunity was recorded in tumour bearing patients if lower lymphocyte concentrations were used.

The increase in cytotoxicity following operation noted in this present study might be a reflection of the removal of tumour derived material with inhibiting properties on cytotoxicity, thereby allowing normal levels of nonspecific cytotoxicity to be expressed. An alternative explanation would be that the lymphocytes were "activated" by the operation by a mechanism apart from actual removal of tumour or the tumour itself might remove "activated" leucocytes and thus prevent their participation in cytotoxic reactions. Nevertheless, the increase in T cell and non T cell cytotoxicity seen in this study indicates that the immunodepression in cancer patients need not be a permanent feature and that even a palliative operation is capable of increasing cytotoxic immune function in metastatic patients. Any relationship between this non-specific increase in cytotoxicity and the "spontaneous" regressions described in hypernephroma remains conjectural.

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Plasma Testosterone Concentrations in Patients with Tumours of the Breast

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Abstract—*The concentration of plasma testosterone in peripheral venous plasma was measured before operation in* 109 *female patients undergoing surgery for a breast lump. The results showed that the levels were not significantly different in those patients with early breast cancer compared with those with benign breast disease. In addition, the values were similar before and after the menopause.*

INTRODUCTION

MUCH work has been reported on the androgen status of women with breast cancer. In particular the levels of androsterone and aetiocholanolone in urine have been found to be subnormal in those women who subsequently develop breast cancer [1]. The abnormality of urinary androgen excretion was present at all ages and was also found in women with advanced breast cancer; it was claimed that those who excreted low amounts of androsterone and aetiocholanolone respond less well to endocrine ablation [2], and that excretion of small amounts of androgen metabolites is associated with a poor prognosis [3]. However, Japanese women excrete less androgen metabolites, but have a lower incidence of breast cancer compared with British women [4].

The sebaceous glands are known to be highly sensitive to androgenic stimulation, and women with breast cancer have been shown to produce increased amounts of sebum [5–7].

The results of the investigations of urinary metabolites do not necessarily conflict with those of sebum production. The urinary metabolites, aetiocholanolone and androsterone, mainly originate from precursors secreted by the adrenalcortices and it is not known which androgens are involved in the stimulation of sebaceous glands. In view of these findings it is perhaps surprising that circulating levels of plasma testosterone have been measured previously in only a small number of patients.

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MATERIAL AND METHODS

Patients

One hundred and nine patients aged between 25 and 70 yr who had been admitted for breast surgery were studied. All gave their informed consent. Blood sampling was performed between 9.00 and 10.00 a.m., as there is evidence of a diurnal rhythm in the level of circulating testosterone [8]. However, the time of the menstrual cycle was not taken into consideration in women who still had menses because the fluctuations are small and the pattern is variable [8]. None of the women were ovariectomized.

The patients were subsequently divided into two groups, those with benign and malignant disease, according to the histology of the breast tissue removed. Only patients with cancer stages I and II of the Manchester classification [9] were included in the group with malignant tumours. The patients were then categorised as being either pre- or postmenopausal, depending on whether 6 months had elapsed since the last menstrual period. The menopausal status of every subject was checked 2 yr later, and if any doubt existed about their original classification these patients were excluded from the study. We regarded the postmenopausal group as being homogeneous as data on plasma testosterone concentrations after the menopause is relatively scarce. It has been shown, however, that there is no significant fall in plasma testosterone concentrations at the menopause [10], and that the ovary continues to secrete significant amounts of testosterone [11], in addition to continuing adrenal production of the hormone [12].

All patients on drugs likely to affect the

concentrations of testosterone in plasma were excluded from analysis. In both pre- and postmenopausal groups the plasma testosterone concentrations of patients with benign breast disease and those with cancer were compared. The levels of plasma testosterone in the premenopausal group were then compared with those in the postmenopausal group.

Plasma testosterone was measured in a liquid phase radioimmunoassay system comprising tritiated testosterone as labelled antigen, and an antiserum raised against testosterone-3-carboxymethyloxime-bovine serum albumen. A mixture of ammonium and calcium sulphates was used to separate the antibody-bound fraction [13]. Under these conditions the cross reaction with 5α-dihydrostestosterone was less than 5%, and the inter-assay variation was within 12%, according to the method of Snedecor [14].

RESULTS

The frequency with which the concentration of testosterone appeared in each group was studied and it was concluded that in general the distribution was best described by the arithmetric mean and range. The values are shown in Table 1. An analysis with Students' *t*-test showed that there was no significant difference between any of the groups.

DISCUSSION

There were no significant differences in plasma testosterone concentrations between patients with benign breast disease and patients with early breast cancer. Our results agree with those of others using an alternative method [15]. Furthermore, Horn and Gordan also found no significant difference in plasma testosterone levels when studying a group with advanced breast cancer [16]. There has been a recent report of significantly raised plasma testosterone levels on sequential sampling in six patients with breast cancer [17]. However, only two of them had early disease and in view of the small numbers involved these results must be viewed with caution.

There was no significant difference between premenopausal and postmenopausal plasma testosterone concentrations. This finding supports the results of others [10, 11], who suggest that the postmenopausal ovary continues to secrete significant amounts of androgens.

Our results do not conflict with the reported findings of reduced levels of androsterone and aetiocholanolone in urine from women with breast cancer, as the metabolites are derived from many precursors in the blood other than testosterone [18]. From our results it is difficult to comment further on the findings of increased sebum production in breast cancer patients, as the role which circulating androgens play in stimulation of the sebaceous glands is not understood. Matters are further complicated by extensive interconversion of circulating androgens in peripheral and growth responsive tissues [19].

Much more must be learned of the androgen status of women with breast cancer, and the physiological role of the different androgens, before reported discrepancies can be explained and their endocrine role in breast cancer understood.

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Table 1. The concentration of plasma testosterone in patients with benign and malignant disease of the breast

	Benign				Cancer				Benign and cancer			
	No. of patients (N)	Mean (pg/ml)	± S.D.	(range)	(N)	Mean (pg/ml)	± S.D.	(range)	(N)	Mean (pg/ml)	± S.D.	(range)
Pre-menopausal	52	372	141	127–660	15	343	101	150–533	67	366	136	127–861
Post-menopausal	13	395	147	181–639	29	383	179	130–775	42	386	170	130–775

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Catechol-O-Methyltransferase in Human Breast Cancers*

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Abstract — *Catechol-O-methyltransferase (EC 2-1-1-6) was identified in human breast tissue. The methyltransferase activity of 78 primary carcinomas and 18 benign tumors was determined. Large variations were observed among the examined normal and pathological breast samples. The relationships between enzyme levels and various pathological aspects of breast tumors were investigated. A statistically significant increase in the enzyme activity was observed in the majority of primary carcinoma samples of higher malignancy, according to Bloom grading. No correlation was found between the catechol-O-methyltransferase activity and the levels of estrogen receptors. Furthermore, enzyme levels were not related to tumoral size, histological type and metastatic involvement. The physiological role of breast catechol-O-methyltransferase, specially in the metabolism of estrogens, is discussed.*

INTRODUCTION

IT IS well established that 2-hydroxylation is an important pathway of estrogen metabolism in both animals [1, 2] and man [3–6]. While 2-hydroxylated estrogens, or catecholestrogens formed fail to exhibit significant uterotrophic activity, it has been recently demonstrated *in vitro* that these compounds have a considerable affinity for the cytosol estrogen receptors from rat pituitary, anterior hypothalamus [7] and rat uterus [8]. These observations suggest that a possible physiological role of these estradiol metabolites might be to function as antiestrogens [8]. In our laboratory a similar interaction of 2-hydroxyestradiol and 2-hydroxyestrone with cytosol estrogen receptors from human breast cancers has been observed (unpublished results). The 2 or 3-methylated derivatives of catecholestrogens do not bind significantly to receptors [8]. 2-methoxy-estrone has been identified as a by product after incubation of either estrone or estradiol with breast tumor supernatant [9–11]. These data lead to conclude that the catechol-O-methyltransferase, enzyme responsible for the O-methylation of catecholestrogens [12], if present in human breast tumor tissues, might be involved in estrogen action on these tissues. We report here unequivocal evidence for the presence of catechol-O-methyltransferase in breast tissue and the relationship between enzyme levels and various pathological aspects of the breast tumors.

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MATERIAL AND METHODS

Reagents

6,7-^{3}H 17 β-Estradiol (specific activity 51 Ci/mmole) and S-adenosyl-L-(methyl-^{14}C)-methionine (specific activity 50 mCi/mmole) were obtained from New England Nuclear Corporation. 6,7-^{3}H-R 5020 (17–21 dimethyl-19 nor pregne 4–9 diene 3–20 dione, specific activity 51 Ci/mmole) was a gift from Roussel-Uclaf. Non radioactive estrogens were purchased from Steraloids, Pauling, NY. The purity of estrogens was checked by thin-layer chromatography just before use.

Tissue preparation

Most tumors examined were obtained from patients subjected to mastectomy. During the operation, samples from the primary tumor and sometimes from axillary lymph metastases and from non neoplastic breast tissue were taken for histological classification and chemical analysis. Within 15 min of excision, all tissues were dissected free of fat, weighed, minced, homogeneized with a Potter and Elvehjem glass-on-teflon homogenizer in 5 vol of ice cold 0.01 M Tris buffer (pH 7.4) containing 12 mM dithiothreitol and 10% of glycerol. The homogenate was immediately centrifuged at 105,000 × ***g*** for 60 min. Aliquots of the supernatant were analysed for progestin and estrogen receptor pro-

teins, catechol-O-methyltransferase activity and protein concentration.

Catechol-O-methyltransferase assay

Catechol-O-methyltransferase activity was determined by measuring the amount of metanephrine from epinephrine and S-adenosyl-(methyl-^{14}C) methionine. Portions of enzyme preparations (100–400 μg protein) were incubated at 37°C for 20 min with 10 μmole of sodium phosphate buffer (pH 7.8), 1 μmole of $MgCl_2$, 0.15 μ mole of epinephrine and 3.6 nmole of S-adenosyl-(methyl-^{14}C) methionine in a final volume of 0.15 ml. The reaction was stopped by the addition of 0.5 ml of 0.13 M borate buffer (pH 10). A blank containing enzyme preparation but no catechol substrate was treated simultaneously for each tissue sample. The formed metanephrine was extracted into 5 ml of ethylacetate. After shaking for 5 min the tubes were centrifuged at 600 ***g*** for 5 min and 2 ml of the organic layer was added to 10 ml of fluid scintillation and assayed for radioactivity.

When 2-hydroxyestradiol was used as the substrate, appropriate concentrations of 2-hydroxyestradiol in 5 μl of ethanol was added to the incubation mixture.

Assay for estrogen and progestin receptors

The presence of oestrogen and progestin receptors were assayed by radioexchange using the method described previously [13].

Assay for protein

Protein concentration was determined according to the method of Lowry *et al.* [14] using crystalline bovine serum albumine as standard.

RESULTS

Catechol-O-methyltransferase activity in breast tissue

Evidence for the identity of the breast tissue O-methyltransferase was obtained by incubating the enzymatic preparations in various conditions (Table 1). The enzymatic activity showed an absolute requirement of Mg^{2+} and was inhibited by tropolone, a potent inhibitor of the catechol-O-methyltransferase [15]. When monophenols or related compounds were used as substrates, no O-methylated products were detected.

To establish the identity of the enzymatically formed product, the radioactive metabolite was extracted into ethylacetate at pH 10 and was evaporated until dryness under nitrogen. The residue dissolved in ethanol was subjected to chromatography:

(1) With epinephrine as substrate, the radioactive product was found to chromatography with metanephrine in paper chromatographic system (butanol-acetic acid–water (4:1:1), *Rf* 0.52).

(2) With 2-hydroxyestradiol the formed methylated product behaved identically with 2-methoxyestradiol on thin-layer chromatography (silica gel-60, chloroform–ethylacetate (3:2), *Rf* 0.63).

Additional evidence for the identity of the breast O-methyltransferase was obtained by using specific antibodies to rat liver purified COMT [16]. The breast enzymatic activity was almost completely inhibited by preincubation with the antiserum (Fig. 1).

Table 1. *Catechol-O-methyltransferase in human breast tissue*

Conditions	Total (cpm)
Blank	620*
Test	13,180
Mg^{2+} omitted	1200
Tropolone, 10^{-4}, added	4190
Substrate:	
Octopamine	0
Tyramine	0
Serotonine	0
O-phosphoethanolanine	0
Histamine	0

50 μl of enzyme preparation were incubated for 20 min with epinephrine or other substrates as indicated. The ^{14}C O-methylated product was extracted in ethylacetate and the radioactivity measured.

*These counts have been subtracted from all of the other results in this table.

These observations indicated that the O-methylating enzyme in human breast tumor is catechol-O-methyltransferase. In order to determine whether the breast tissue catechol-O-methyltransferase could be distinguished from the liver enzyme, some biochemical properties of both enzymes were compared. Both enzyme preparations had similar pH optima (7.8–8.3) and an optimal concentration of Mg^{2+} (1 mM). The apparent K_m values determined by double reciprocal Lineweaver–Burk plots were similar for both enzymes: 2.2×10^{-4} M and 1×10^{-5} M respectively for the substrates, epinephrine and 2-hydroxyestradiol (Fig. 2). The low K_m value found for 2-hydroxyestradiol is of the same magnitude as values reported by Ball *et al.* for human liver catechol-O-methyltransferase ($K_m = 1.4 \times 10^{-5}$ M) [17].

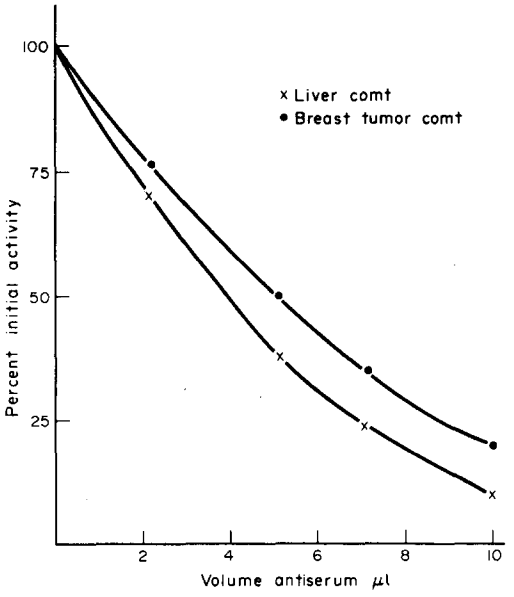

Fig. 1. Inhibition of catechol-O-methyltransferase from human liver and breast tumor by rabbit antiserum to rat liver enzyme. Enzyme preparations were pre-incubated with 10 μmole *of sodium phosphate buffer,* 1 μmole *of* $Mg\ Cl_2$ *and varying amounts of antiserum or control rabbit serum. After* 15 min *at* 37°C, 0.15 μmole *of epinephrine and* 3.6 nmole *of* ^{14}C*-SAM were added to assay residual enzyme activity.*

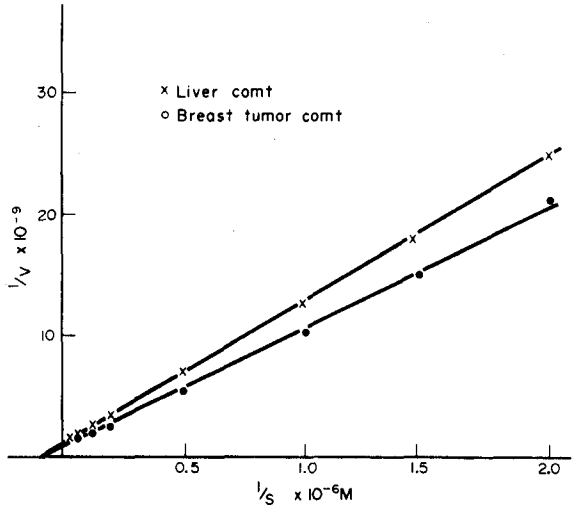

Fig. 2. Determination of K_m *values for 2-hydroxyestradiol and catechol-O-methyltransferase from breast tumor and liver. Enzyme preparations were incubated for* 20 min *with appropriate concentrations of substrate and* 2.4×10^{-5}M *of labeled S-adenosyl-*L*-methionine.*

Catechol-O-methyltransferase in primary and metastatic breast cancer

The catechol-O-methyltransferase activity was found to be quite variable in human breast tissue. The standard error of the mean was in the order of 20–25% of the mean in "normal" tissues as well as in diseased tissues of 32 cases where normal tissue and malignant tumor were obtained from the same patient: 27 (84%) showed a 2–30 fold increase for catechol-O-methyltransferase activity in carcinoma samples when compared to "normal" tissues. In 5 cases, levels were found similar.

The mean values for enzyme activity in normal breast tissue and benign and malignant tumors are given in Table 2. The histologically "benign" tumors consisted of 10 fibroadenomas and 8 cystosarcoma phyllodes. No significant difference was observed between normal breast tissue and benign tumors. For carcinomas, the catechol-O-methyltransferase activity was compared to the histologic grade of the tumor using the grade I–III system of Bloom and Richardson [18]. Activity in grade I tumors was not significantly different from that obtained in normal tissue (Table 2). On the contrary, a statistically significant increase of catechol-O-methyltransferase was found to exist in grade II and III lesions.

Table 2. Catechol-O-methyltransferase activity in normal and abnormal human breast tissues

Tissue	No. samples	COMT activity (mean ± S.E.M.)
Normal breast	32	344 ± 83
Benign tumor	18	570 ± 155*
Carcinoma (Grade I)	13	555 ± 152*
Carcinoma (Grade II)	32	1538 ± 328†
Carcinoma (Grade III)	33	1716 ± 450‡

COMT activity is expressed as pmole of radioactive metanephrine formed per 20 min per mg of cytosol protein.
*Not significantly different ($P < 0.02$—Student's *t*-test) compared with normal breast.
†Significantly different ($P < 10^{-9}$—ε test) compared with normal breast.
‡Significantly different ($P < 10^{-8}$—ε test) compared with normal breast.

Sixty-six malignant tumors were analyzed for a possible correlation between catechol-O-methyltransferase activity and estrogen and progestin-binding capacity. The results are summarized in Fig. 3. There was no relationship between the enzyme activity and the presence or absence of receptors (< 100 fmole/g tissue). In addition, no difference in levels of catechol-O-methyltransferase was observed in relation to size of primary tumor, hystological type of carcinoma (colloid, medullary, lobular, tubular), and number of metastatic lymph nodes. Finally, there was no influence of menopausal status of patients on the catechol-O-methyltransferase activity in breast malignant tissue.

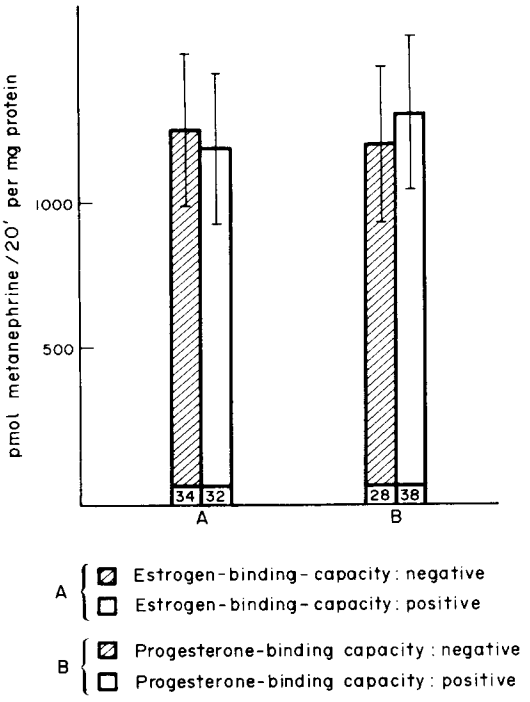

Fig. 3. Relationship between estrogen and progesterone binding capacity of breast tumors and catechol-O-methyltransferase activity. Values are presented as mean ± S.E.M. Number of samples examined is shown at the base of each bar.

The results of the lymph nodes examinations are presented in Table 3. In all cases examined, catechol-O-methyltransferase activity was higher in lymph nodes containing metastases compared to "normal" lymph nodes. Table 4 lists data obtained in normal and invaded lymph nodes from the same patient. There was a relationship between the enzymatic activity and the degree of metastatic involvement. A similar increase was observed in breast carcinoma compared to "normal" tissue and benign tumor (cystosarcoma phyllodes) from the opposite breast in the same woman (Table 4).

Table 3. Relationship between the presence or absence of lymph node metastases and the catechol-O-methyltransferase activity

	COMT activity (pmole/20'/mg protein)	
Patient	Normal lymph nodes	Metastatic lymph nodes
RIG...	320	
MIC...	630	
MAR...	910	
IZA...	100	
NAA...	770	6675
GUI...	100	8270
GAR...		1150
ELL...		1950
RAN...		2370
MOU...		3040
DUR...		1620
VAR...		2460
THI...		8150
NAN...		3200
QUA...		2100
BER...		7340

Table 4. Catechol-O-methyltransferase activity in normal and abnormal tissues from the same patient

Patient	Normal tissue	Cystosarcoma phyllodes (right breast)	Adenocarcinoma (grade II) (left breast)
Bor...	695	680	3215
	Normal lymph node	Lymph node with low metastatic involvement	Lymph node with massive metastatic involvement
Gut...	100	535	8270

COMT activity is expressed as pmole of radioactive metanephrine formed per 20 min per mg of cytosol protein.

DISCUSSION

The experiments described in this report show the presence of catechol-O-methyltransferase in human breast tissue. In contrast, other methyltransferase enzymes (phenol-O-methyltransferase enzymes (phenol-O-methyltransferase, non specific N-methyltransferases, histamine N-methyltransferase) could not be detected in this tissue. The cathechol-O-methyltransferase activity was found to be very variable among the examined breast samples. Similar variations in levels of different enzyme activities in human breast tissue have been reported [19]. Moreover, an important aspect of studies on the estrogen binding capacity of breast carcinomas relates to considerable tumor to tumor variations in extrogen receptor levels. In spite of this great variability in the catechol-O-methyltransferase activity and although the values extended over a broad range, a significant increase in the activity of this enzyme was observed in the majority of primary carcinoma samples of higher malignancy (grade II and III). The mean value was approximately 5 fold that found in normal breast tissue. In contrast, statistical analysis failed to disclose a significant difference in grade I tumors and benign lesions when compared to normal tissues. We must emphasize the fact that the enzyme activity was expressed per mg cytosol protein. Although there was essentially no difference in protein content among the tissues examined, it is obvious that the expression of enzyme activity per mg DNA would normalize any difference in enzyme activity that would be due to difference in cell number. The DNA content have been reported

to be significantly increased in fibroadenoma and infiltrating ductal carcinoma, when compared to normal breast [19]. However, it appears doubtful that as high as 30 fold increases for catechol-O-methyltransferase could be due only to cellularity increase. So, the cellular pleomorphism of many carcinomas may be responsible for large variations in the catechol-O-methyltransferase content of breast tissue. Cytological studies with fluorescent labeled antibodies should provide an answer to this problem.

The question arises, what can be the physiological significance of the presence of catechol-O-methyltransferase in breast tissue? 2-Methoxyestrone has been identified on column chromatography and on thin-layer chromatography after incubation of either estrone or estradiol with human breast tumor supernatants [11]. This finding strongly suggests that breast tissue catechol-O-methyltransferase is involved in estrogen metabolism. As postulated by Martucci and Fishman [8], its possible physiological role in breast tumor might be to abolish the catechol-estrogen binding ability to tumor cytosol estrogen-receptors. However, data reported here demonstrate the lack of correlation between the levels of catechol-O-methyltransferase activity and the estrogen binding ability of breast tumors. Furthermore, unlike earlier observations [11], attempts for carrying out a complete identification of methylation products after incubation of human breast tumor with estrone or estradiol have been unsuccessful. Therefore, further studies are required to bring a direct evidence to the presence of a 2-hydroxylase activity in breast tissues. By now, preliminary results suggests that catecholestrogens formation, if present in breast tumors, is in no way related to the apparent capacity of O-methylation in these tissues.

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Comparison of Soluble Oestrogen and Progestin Receptor Content of Primary Breast Tumours from Japan and Britain

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Abstract — *Soluble oestrogen (R_E) and progesterone (R_P) receptors have been measured in primary breast tumours from English and Japanese women. The only difference between the two groups of patients was that the Japanese had a higher proportion (31%) of R_E negative tumours than the English group (19%).*

INTRODUCTION

THE PROGNOSTIC significance of oestrogen receptor (R_E) measurements in breast tumours is of increasing interest in relation to hormone therapy [1, 2]. All of the reports published thus far have been concerned with R_E measurements in tumours from countries with a high incidence of breast cancer. In view of the low incidence of breast cancer in Japanese women it was of interest to see if the R_E content of their tumours differed from that of tumours from British women. We have carried out such a comparison on tumours from the National Cancer Center Hospital in Tokyo and from the Breast Unit at Guy's Hospital, London. Progesterone receptors (R_p) were also assayed.

MATERIAL AND METHODS

Tumours from Tokyo were stored in liquid nitrogen for a maximum of 8 weeks and dispatched to London by air in an insulated container with solid carbon dioxide. They were then stored in liquid nitrogen for a maximum of 2 weeks prior to assay. Four separate batches of tumours were processed in this way. The London tumours were transported in solid carbon dioxide from the operating theatre to the laboratory and were stored in liquid nitrogen for a maximum of 2 weeks prior to assay.

A known weight of tumour slices was pulverized in a microdismembrator (Braun Instruments, Melsungen) using teflon containers pre-cooled in liquid nitrogen. The tumour powder was stirred in 8 vol of 5 mM sodium phosphate, 10% v/v glycerol, 1 mM thioglycerol pH 7.4 [3] for 10 min at 4°C and centrifuged for 10 min at 1500 ***g***. The supernatant was separated from the pellet and any fat at the surface and used for the R_E and R_p assays. Aliquots were taken for protein estimation [4].

R_E assay

All tubes contained 200 µl supernatant plus 20 µl 10^{-8} M 2, 4, 6, 7^3H oestradiol (82 Ci/m mole, Radiochemical Centre, Amersham). Duplicate tubes contained 20 µl 10^{-5} M diethylstilboestrol whilst control tubes contained the same volume of buffer. After overnight equilibrium at 4°C, 240 µl of dextran/charcoal suspension (D/C 0.25% charcoal, 0.0025% Dextran T20 in 10 m M Tris: 1 mM EDTA, ph 7.4) was added and, after mixing, was allowed to stand at 4°C for 90 min [5]. After centrifugation at 10^3 ***g*** for 10 min, 300 µl of the clear supernatant were added to 5 ml phosphor (5 g diphenyloxazole/l toluene). The mixture was shaken, stood at room temperature for at least 1 hr, cooled to 4°C and counted in a Packard Tricarb Scintillation counter. Quenching was corrected for by external standardization. Specific binding was measured as the difference between the control and diethylstilboestrol-treated samples.

R_p assay

All tubes contained 200 µl supernatant plus 20 µl 10^{-7} M 1, 2, 6, 7^3H progesterone (84 Ci/mmole, Radiochemical Centre, Amersham) plus 20 µl 10^{-5} M cortisol. Triplicate

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tubes contained 20 μl 10^{-5}M norethisterone whilst control tubes contained the same volume of buffer. The samples were processed as described for R_E except that D/C treatment was limited to 10 min. Specific binding was calculated as the difference between the control and norethisterone-treated samples.

With both R_E and R_P assays, the lower limit of assay sensitivity was 3 fmole/mg protein: tumours with less than this value were classified as negative. All results were obtained with primary tumours.

RESULTS

A comparison of R_E levels in Japanese and British patients is shown in Fig. 1(a). The

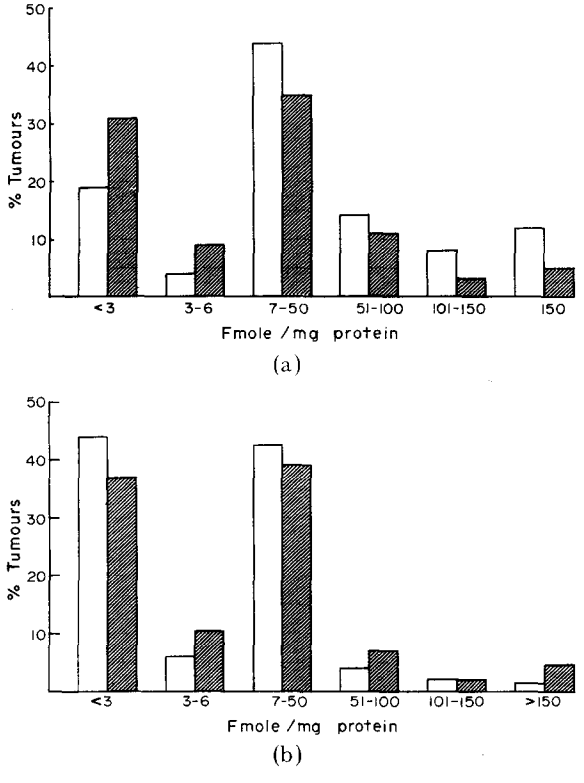

Fig. 1. Percentage of tumours containing different levels of R_E (Fig. 1a) and R_P (Fig. 1b). Unshaded columns: British tumours, total number R_E 136; R_P 87. Shaded columns: Japanese tumours, total number R_E 65, R_P 47.

Japanese group contain a higher proportion of negative tumours than the British group (χ^2 test, $P<0.001$). In other respects, the two populations are similar. Four separate batches of Japanese tumours were analysed and the proportion of R_E negative tumours was similar in each batch (range 29–35%). Five separate batches of British tumours gave a range of 12–32% negative tumours. The slightly higher proportion of negative Japanese tumours could be due to different storage conditions. R_E content of tumour tissue is stable for months in liquid nitrogen and for several weeks in solid carbon dioxide. Hence, storage alone is unlikely to be a cause but it is impossible to eliminate unknown accidents occurring during transit. However, such a hypothetical accident would have had to happen to all four batches of Japanese tumours; also, one batch of tumours had the same proportion of R_p negative tumours as the British tumours. If storage conditions do not provide the explanation it would be tempting to speculate that the Japanese might have fewer unresponsive but R_E positive tumours; clinical correlations would be required to answer that possibility.

Only two batches of Japanese tumours were analysed for R_p content but the profiles of R_p content were similar in Japanese and British tumours (Fig. 1b).

The distribution of R_E and R_p levels in tumours from patients of different ages is shown in Figs. 2(a) and 2(b) respectively. With one exception, no significant differences are evident between the Japanese and British tumours. The exception is in the R_E levels in the 60–69 yr-old group in which the British tumours exhibit a higher R_E content than the Japanese (Mann–Whitney U test, $P<0.005$). No explanation can be offered for this result. The small number of tumours from Japanese patients in the higher age range conforms with the known incidence pattern in Japan.

The number of combined R_p and R_E measurements made on Japanese tumours is small but comparison of tumours containing the various combinations of R_p and R_E (Table 1) indicates that, with the possible exception of the small group of R_E-, R_p+ tumours, no difference exists between the tumours from the two countries. With the possible exception of the small number of R_E-, R_p+ tumours, the percentage of British tumours in the groups shown in Table 1 is similar to that reported for

Table 1. Combined oestrogen (R_E) and progestin (R_p) assays on breast tumours

	(%) Tumours	
	British	Japanese
R_E+, R_p+	40	37
R_E+, R_p-	25	24
R_E-, R_p-	24	20
R_E-, R_p+	10	20

These data are based on 87 British and 46 Japanese tumours. Tumours containing < 3 fmole/mg protein have been classified as −; all others are +.

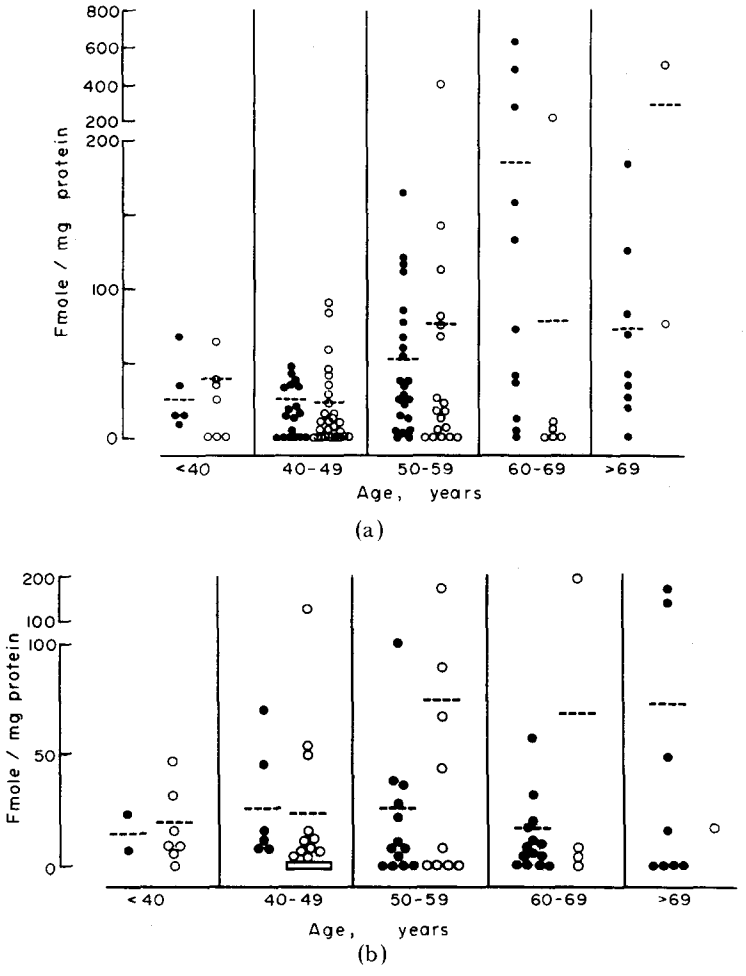

Fig. 2. R_E *(Fig.* 2a*) and* R_p *(Fig.* 2b*) levels of tumours as a function of age of patient.*
●, *British patients;* ○, *Japanese patients.*
The dashed line is the mean value for the positive (> 3 fmole/mg *protein) tumours within any one age group. There were* 13 R_p *negative Japanese tumours in the* 40–49 yr-*old group. These are represented as an open rectangle.*

American tumours by McGuire [6]. Of the R_E+, R_p+ tumours, no difference in R_p:R_E ratios was detected between the Japanese and British tumours.

DISCUSSION

The only major difference in R_E and R_p content of primary Japanese and British breast tumours was that the former had a slightly higher proportion of R_E negative tumours than the latter group. This might be due to different storage conditions of the two groups of tumours but it could also have clinical significance.

It is not known whether the response rates to the endocrine treatment of advanced breast cancer differs in Japanese and British patients although, as far as adrenalectomy and oophorectomy are concerned, the results look very similar between Japan and the West [7]. A prospective study is being undertaken to compare the response of British and Japanese women with advanced breast cancer to castration, androgen or oestrogen therapy.

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Analysis of Dehydroepiandrosterone and Androstenediol in Human Breast Tissue Using High Resolution Gas Chromatography–Mass Spectrometry

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Abstract—*Dehydroepiandrosterone (DHA) and 5-androstenediol have been measured in human breast tissue by a method consisting of extraction and purification with final analysis by gas chromatography–mass spectrometry. The method has been shown to be specific and reproducible, measuring only free DHA and not its sulphate. Preliminary results indicate that levels of DHA are in the range* 15–200 ng/g *tumour whereas in fibroadenomata the amounts are much higher* (200–1000 ng/g). *Androstenediol is present in tumours and benign tissues in similar amounts* (10–200 ng/g).

INTRODUCTION

THE RELATIONSHIP of C_{19}-steroids to the responsiveness of breast tumours to endocrine therapy has been the subject of many studies and a number of groups have reported that the estimation of 11-deoxy-17-oxosteroids in urine samples from their patients with breast cancer has been of value in discriminating between those who will and those who will not benefit from adrenalectomy or hypophysectomy [1–3]. Although similar investigations from our laboratories [4] supported the view that steroid excretion in many patients with breast cancer differed from the normal population, the results did not agree completely with those previous reports, abnormalities being found only in patients with advanced cancer of the breast.

Further studies from these laboratories [5, 6] and from others [7] were directed to the paraendocrine behaviour of breast tumour tissue and its capacity to metabolise plasma C_{19}-steroids, particularly dehydroepiandrosterone sulphate (DHA sulphate) and DHA, to compounds such as testosterone, androst-5-en-3β,17β-diol (androstenediol), 17β-hydroxy-5α-androstan-3-one and oestrone. Obviously, the C_{19}-steroid concentration in the breast tumour may well play a role in regulating the oestrogenic stimulation of this tissue and Poortman, Prenen, Schwarz and Thijssen [8] have reported that androstenediol will displace oestradiol-17β from the cytoplasmic receptor in breast tumours. Concurrent studies from this Institute indicate that a 50-fold excess of androstenediol will result in approximately 50% displacement of oestradiol-17β [Powell-Jones, submitted for publication].

As part of this investigation into the role of C_{19}-steroids in breast cancer, the present communication deals with the assay of DHA and androstenediol in breast tissue. The procedure is based on a simple extraction and purification with quantitation by gas chromatography–mass spectrometry using high resolution selected ion detection and internal standards to monitor losses during extraction.

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MATERIAL AND METHODS

Chemicals

DHA and androstenediol were obtained from Sigma Chemical Co., London; 3α-hydroxy-5-androsten-17-one and 5α-androstanediol (5α-androstane-3α,17α-diol) from Steraloids Inc., U.S.A. Bis (N, O) trimethylsilyl acetamide (BSA) was purchased from Jones Chromatography, Llanbradach, Mid-Glam., U.K. and polyfluorokerosene from Koch-Light Ltd., England. All solvents were of analar grade and were distilled before use.

Tissues

Breast tissue, received from a number of centres, was homogenised by powdering in a Thermovac automatic frozen tissue pulveriser (Telcolab Corpn., N.Y.) and then suspended as a 15% (w/v) homogenate in 10 mM Tris-HCl buffer pH 7.4 containing EDTA (1 mM) and sodium azide (3 mM), using a glass–glass homogeniser. Portions of the homogenate were stored at −20°C until extracted.

Extraction of steroids

Internal standards, 3α-hydroxy-5-androsten-17-one (20 ng) and 5α-androstanediol (5 ng), in ethanol, were added to a stoppered glass tube followed by 0.5–2.0 ml of homogenate. After mixing vigorously the tubes were left at 4°C overnight.

Lipid material was extracted twice with ethanol/acetone (1:1, v/v) (5 ml) by agitation for 2 min on a vortex mixer, and the combined extracts evaporated to dryness under a stream of nitrogen in a water bath at 40°C. Most of the non-steroidal lipid was then removed using a modification of the method of Ismail, Love and McKinney [9]. Ethyl acetate (50 μl) was added to the residue followed by 1% aqueous $CaCl_2$ solution (5 ml) and the tubes placed in a water bath at 50°C for 10 min without shaking. The tubes were then carefully removed, placed in ice for a further 10 min and then slowly filtered through a scintered glass funnel (porosity 1). The funnels were washed with ice-cold $CaCl_2$ solution (1 ml).

The filtrate was extracted twice with diethyl ether (5 ml) and the solvent evaporated to dryness under nitrogen. Residues were transferred in a small volume of ethanol to tubes for derivative formation.

Derivative formation

Trimethylsilyl ethers of the steroids were prepared by drying the tubes *in vacuo* over phosphorus pentoxide for 1 hr and adding BSA (20 μl) to the residue. The tubes were tightly stoppered, left at room-temperature overnight and the derivatised extracts analysed within 48 hr.

High resolution molecular ion monitoring

Details of the procedure by these laboratories for selected ion monitoring at high resolution have been described [10, 11]. A Varian 2700 gas chromatograph fitted with a 2 m × 2 mm i.d. helical glass column containing 3% OV-17 on Gaschrom Q (100–120 mesh) was interfaced to a Varian MAT 731 mass spectrometer by a two stage Watson–Biemann type separator followed by a glass lined probe. The ion source temperature was 150°C and the gas chromatograph injector, separator and probe temperatures were 275°C, 250°C and 245°C respectively. The electron beam energy was 70 eV with the resolving power at 8500–10,000 (10% valley) at the mass to be monitored, which was located with the aid of the reference compound polyfluorokerosene. In all cases the most intense peak was the molecular ion of the steroid derivative, and this was the ion monitored. The steroid extract (1–2 μl) was injected using BSA as solvent.

Determination of results

The output of the mass spectrometric detector was displayed on a potentiometric recorder and the respective peak heights of the steroid and internal standard were measured and compared with a standard curve for quantitation. DHA and androstenediol were assayed in the same extract in two separate injections.

RESULTS AND DISCUSSION

In order to show that there was little likelihood of any endogenous material interfering with the peak associated with the internal standards selected, a number of breast tissues (normal, benign and neoplastic) were extracted with no steroids added. In no case was there found a significant peak with the same ion mass nor a gas chromatograph retention time identical with the derivative of 3α-hydroxy-5-androsten-17-one or 5α-androstanediol. For the assay of DHA the ion mass monitored was 360.24845 and a typical trace of one standard is shown in Fig. 1(a), demonstrating an adequate gas chromatographic separation of the two compounds. In the majority of tissue extracts a peak of unknown material with this mass was found, but this had a very much shorter retention time on the column (Fig. 1b).

For the androstenediol assay it was necessary to monitor at an ion mass of 436.3192 and then at 434.30364 for, respectively, 5α-androstanediol and androstenediol derivatives. Figure 2 shows typical traces of a standard (Fig. 2a) and of a tissue extract (Fig. 2b).

Standard curves were set up with every group of tissues extracted and a linear relationship was found between the ratio of steroid to internal standard and the amount of steroid, both for DHA (Fig. 3) and androstenediol (Fig. 4). Although the between-assay variation in stan-

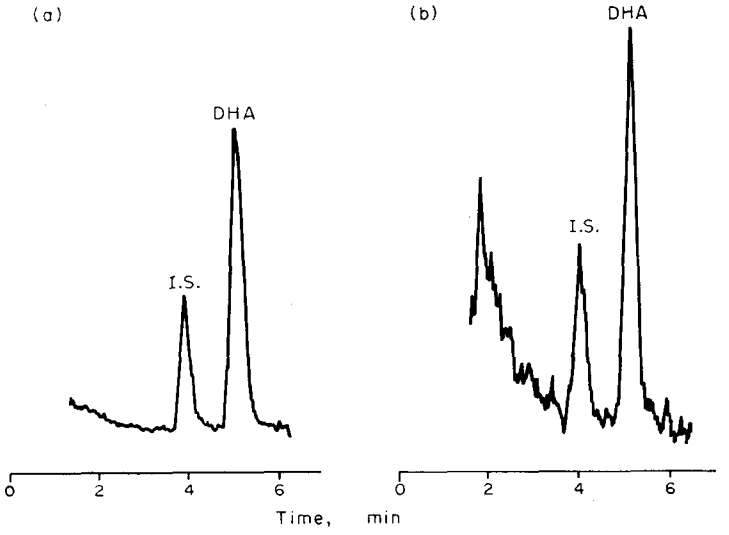

Fig. 1. *Separation and detection of dehydroepiandrosterone and* 3α*-hydroxy-*5*-androsten-*17*-one as the trimethylsilyl derivatives by gas chromatography–mass spectrometry.* (a) *Typical trace of pure standards,* (b) *Typical trace of a tissue extract.*

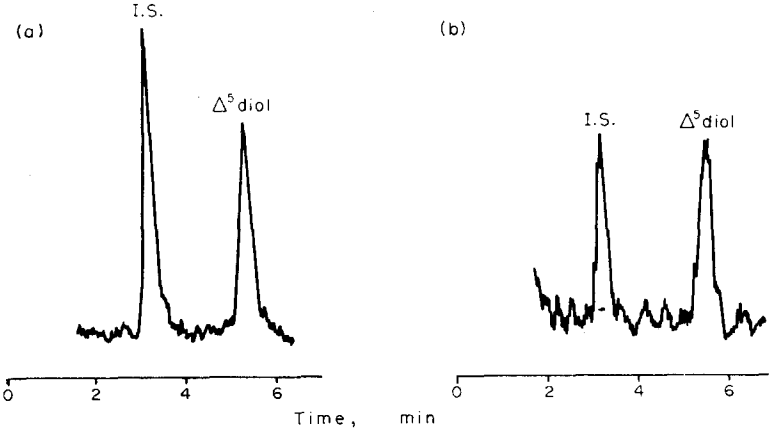

Fig. 2. *Separation and detection of androstenediol and* 5α*-androstane-*3α, 17α*-diol as the trimethylsilyl derivatives, by gas chromatography–mass spectrometry. Ion mass* 436.3192 *monitored for internal standard and* 434.30364 *for androstenediol.* (a) *Typical trace of standard,* (b) *Typical trace of a tissue extract.*

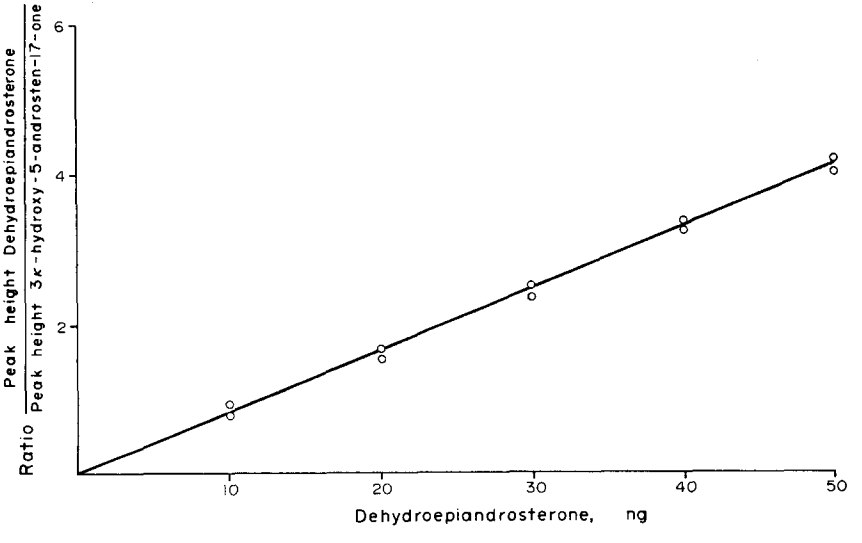

Fig. 3. *Typical calibration curve for dehydroepiandrosterone assay.*

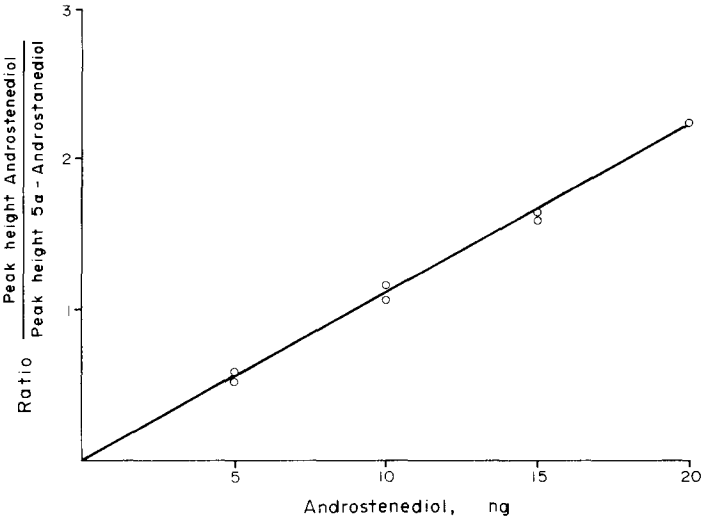

Fig. 4. Typical calibration curve for androstenediol assay.

dard curves was small, it was considered necessary to include a curve with each extraction batch.

A number of experiments were performed to test the efficiency of extraction of DHA and androstenediol from tissues. No differences were found between the steroid content of extracts of tissue prepared as described or when tissue was extensively homogenised with the acetone/ethanol mixture. Furthermore, the efficiency of the extraction procedure did not depend upon the amount of tissue present up to 300 mg (2 ml of homogenate) as shown in Table 1.

Table 1. Endogenous DHA and androstenediol in different quantities of 15% homogenate of a primary breast tumour

Amount of homogenate (ml)	Steroid content (ng) DHA	Androstenediol
2.0	18.5	3.5
1.5	14.5	2.4
1.0	10.0	1.8
0.5	5.5	—*
0.0	0.0	0.0

*Derivatised extract hydrolysed before analysis completed.

Because of the high levels of DHA sulphate in plasma, it is possible that some of the DHA detected could have come from contaminating plasma, following hydrolysis of the sulphate during processing. Table 2 shows the DHA measured in samples of tissue from two fibroadenomata, generally more vascular than malignant tissue, following the addition of 35 ng of either DHA or its sulphate before extraction. It can be seen that whereas recovery following the addition of the free steroid is good, the sulphate contributed nothing to the measurement. Therefore the method estimates only the free steroid content.

Table 2. DHA measured in 1 ml portions of a 15% homogenate of two fibroadenomata either alone or following addition of free DHA (35 ng) or of its sulphate (35 ng)

Additions	Dehydroepiandrosterone (ng)	
	Fibroadenoma 1	Fibroadenoma 2
None	3.5	18.5
DHA (35 ng)	35.0	53.8
DHA sulphate (35 ng)	4.0	17.5

The reproducibility of the assay obviously will depend largely upon the homogeneity of the prepared sample, and breast tissue is well known to be difficult to homogenise. Table 3 shows the values obtained following the extraction of five 1 ml aliquots of a 15% homogenate of a fibroadenoma. The one sample with low levels of both steroids probably demonstrates the heterogeneity of the homogenised preparation before extraction.

Table 3. DHA and androstenediol in replicate 1 ml portions of a 15% homogenate of a fibroadenoma

	DHA	Androstenediol
	(ng)	(ng)
	52.7	8.0
	56.2	8.5
	34.5	7.8
	51.7	9.0
	52.3	8.0
Mean	49.5	8.3
Coefficient of variation	17	6

In this pilot study on the amount of DHA and androstenediol in breast tissues, a small number of malignant tumours and fibroadenomata have been assayed. As shown in Table 4, the concentration of these steroids is sufficiently high to be accurately determined in both types of tissue. Although the levels of DHA in the benign condition appear to be considerably higher than in the malignant tumours, it should be pointed out that these results are not corrected for cellularity, either by protein or by DNA estimation. Further work is necessary to determine whether such analyses can be related to subsequent development of the disease or to its clinical management.

Acknowledgements—The authors are grateful to the Tenovus Organisation in Cardiff and to the M.R.C. (Grant No. G974/125C) for financial support for this work.

Table 4. DHA and androstenediol content in primary breast tumours and fibroadenomata

Tumours	DHA	Androstenediol
	(ng/g)	(ng/g)
40–1	16.0	16.7
40–3	26.7	
41–2	46.7	22.0
42–3	36.7	53.3
28–6		16.7
34–10	40.0	13.3
39–1	44.7	26.0
33–4	233.3	200.0
Fibroadenomata		
36–1	~ 366.7	57.3
28–3	~ 733.3	
36–2	~1000.0	42.0
43–6	245.3	134.7
28–4	330.0	55.3

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Combined Therapy of the Spontaneous Mouse Mammary Tumour: Methotrexate and Hyperbaric Oxygen Irradiation

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Abstract—*The response of spontaneous mammary adenocarcinoma of female C_3H/Bts mice to combinations of methotrexate administration and irradiation with* 14 MeV *electrons under hyperbaric oxygen* (3 *ata*) *was measured. The wide variation in doubling times of these tumours was taken into account by making up experimental groups to contain equal numbers of faster- and slower-growing tumours. A standard dose of* 80 mg/kg *methotrexate injected into the tumour, followed by citrovorum rescue* 24 hr *afterwards, produces a small but significant tumour response. There was no effect on the response of tumours of* 1000 rad *under HPO when methotrexate was given* 7 days *before irradiation, but a significant increase in response, which was essentially additive, when the drug was given as a* 24 hr *pretreatment. Oxygen concentration measurements showed there to be no essential difference in rate or degree of oxygenation between pretreatment and control groups.*

A dose of 3000 rad *under HPO was followed* 7 days *later by methotrexate administration, and gave an increased effect on the tumours which was more than additive. Early skin reactions were essentially the same for groups given combined treatment or irradiation only.*

Possible mechanisms involved, long term survival of the mice and metastatic spread from the primary tumour are discussed.

INTRODUCTION

THE EFFECTIVENESS of combined radiotherapy and chemotherapy compared with that of radiotherapy alone is still a subject of clinical trial. Combined therapy appears to produce better results than radiotherapy alone in the treatment of childhood leukaemias [1], bronchogenic carcinoma [2], oat cell carcinoma of the lung [3], lymphomas [4] and perhaps in some solid tumours of the head and neck [5, 6]. There are many questions outstanding, not least that of the risk of increasing the incidence of toxic side effects [7] with combined therapy. The work described here uses an animal tumour system as a model to investigate the effectiveness of combining the antifolate agent, methotrexate, with irradiation in different time relationships.

A dose of 1000 rad under hyperbaric oxygen (HPO) was used as a test dose for combination therapy with methotrexate, where the drug was given *before* irradiation. The drug was administered at a dosage of 80 mg/kg, 7 days or 24 hr pre-irradiation. The initial choice of this test dose of radiation was made in the expectation that it would not be a tumour "cure" or "control" dose, but that it would produce a marked tumour response, since synergistic or additive effects cannot be demonstrated on maximal response.

In further experiments where the effect of administering the drug *after* the radiation treatment was to be studied, it was decided to use a larger standard dose of irradiation—3000 rad under HPO. Although this was still not a "cure" dose, it would make it possible to evaluate the effects of the combined treatments on two endpoints rather than one, namely both tumour and skin responses.

As the irradiations were carried out under hyperbaric oxygenation, it was necessary to establish whether or not methotrexate pretreatment could alter the degree of oxygenation of the tumour. A series of investigations was made using oxygen cathode electrodes to measure directly tumour oxygen concentrations in

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mice treated with methotrexate, but not irradiated.

MATERIAL AND METHODS

1. *Tumour response*

The tumour used was the spontaneously arising mammary tumour of the female C_3H mouse. Mice bearing mammary tumours in the thoracic region were withdrawn from the C_3H/Bts stock colony and caged 8 to a box; they were fed Dixons CDDM diet with water *ad libitum*. Tumour diameters were measured three times a week, and the relative tumour volume (the product of 3 orthogonal dia) plotted on a semilogarithmic plot against time. From this the volume doubling time can be calculated, and the response of the tumour to either drug or radiation treatment measured by the formula for assessing radiation response described by Cheshire and Lindop [8], where tumour response =

$$\frac{\text{time taken to regrow to volume at treatment}}{\text{post treatment doubling time}}$$

The variable doubling time of the spontaneous C_3H mammary tumours make the use of this type of assessment essential [9].

After treatment each mouse was caged separately. They were examined daily and tumour measurements were continued until the death of the animal. Since the C_3H mouse often produces more than one mammary tumour, it was sometimes necessary to kill an animal if second or subsequent tumours caused great discomfort. The ages of the mice at irradiation ranged from 11 months to 26 months old with a mean age of 20 months.

2. *Irradiation*

Tumours were irradiated with a single dose of electrons of 14 MeV mean energy from the linear accelerator in the Department of Radiotherapy, St. Bartholomew's Hospital. Irradiations were carried out under 3 ata oxygen, in a pressure tank designed [10] to allow the pressurization of 4 mice simultaneously with consecutive irradiation of each mouse in turn. The beam of electrons was collimated outside the tank by a 11.5 cm Dural applicator and internally by a 5 cm thick lead collimator with a hole 2.2 cm dia. Mice were anaesthetized with 65 mg/kg sodium pentobarbitone solution i.p. and placed on a Perspex platform fitted beneath the internal collimator. The platform was adjusted so that the tumour lay concentrically with the collimator hole. A strip of 35 mm photographic film (Ilford Fine Grain Safety Positive) in black polythene was placed under each tumour, to check that the tumour was central in the field during irradiation, and a LiF capsule was fixed in the field adjacent to the tumour. An ionization chamber in the beam outside the pressure tank was calibrated against a 0.2 cm^3 Baldwin Farmer Sub-Standard Dose Meter in the position of the tumour, and the chamber voltage produced by the required dose determined. During the exposure of the mice the ion chamber voltage was fed to a trigger-comparator unit which stopped the machine when this set value was reached.

3. *Hyperbaric oxygenation*

Anaesthetized mice were compressed to 3 ata—30 lbf in^{-2} gauge pressure (207 kPa) in the pressure tank at 30°C. Humidifiers were placed in each mouse compartment, and CO_2 build-up prevented by the 6 l/min O_2 leak. The pressure in the tank was raised by 5 lbf in^{-2} (34.5 kPa) increments over 2 min and the mice maintained at 30 lbf in^{-2} (207 kPa) for 4 min before irradiation for washout. (The timing for equilibration at different pressures was confirmed by oxygen cathode electrode studies, see below.) After the 4 mice had been irradiated consecutively, the tank was decompressed over a period of 1.5–2 min. The total time spent under pressure therefore did not exceed 16 min. No mice died as a result of compression, except for 2 in the first experiment which were shown at *post mortem* examination to have substantial pulmonary metastatic deposits of mammary tumour cells.

4. *Methotrexate administration*

A single dose of 80 mg/kg methotrexate (Methotrexate sodium, Lederle) injected directly into the C_3H mammary tumour will produce a small but positive tumour response [9]. This dose of methotrexate followed 24 hr later by 200 mg/kg citrovorum factor (Calcium leucovorin, Lederle) s.c. was shown to be the optimum dose for tumour response to methotrexate at a level of toxicity where all the mice could be saved by citrovorum. Intratumour injection of methotrexate produced a more consistent effect on the tumour, with a satisfactory dose–response relationship over a range of doses from 20 mg/kg to 80 mg/kg without citrovorum rescue [9]. When methotrexate was injected intraperitoneally without citrovorum there was no dose–response relationship, with high mortality at the higher doses. Control mice were given equal volumes of sterile saline solution, injected into the tumour.

5. *O_2 Concentration measurements in tumours*

Oxygen concentration was measured using 200 μm gold oxygen cathode electrodes of the "open" type, insulated with an araldite mixture except at the tip. A reference voltage of 0.6 V was applied via a silver/silver chloride electrode. Currents proportional to oxygen concentration were amplified and recorded on a multi-channel apparatus [11]. Absolute values of oxygen concentration and electrode drift were determined by pre- and post-calibration in aerated saline at 37°C.

Female C_3H/Bts mice with spontaneous mammary tumours arising in abdominal or inguinal sites and not therefore suitable for irradiation experiments were used for the oxygen concentration measurements. (The histopathology of tumours arising on the abdominal milk crest does not differ from that of tumours arising in the thoracic region, and there is no reason to suppose that the site of the mammary tumour along the milk crest alters its response to irradiation or oxygenation. The similarity of response to oxygenation of both axillary and inguinal mammary tumours has been demonstrated in experiments carried out in this laboratory.) The tumour volumes were comparable with those irradiated, i.e., about 1000 mm^3.

The mice, anaesthetized with 65 mg/kg body wt sodium pentobarbitone as for the irradiation experiments, were lightly tethered to a Perspex platform. One or two calibrated oxygen cathodes were inserted into the tumours, a hypodermic needle being used to facilitate their entry through the skin. The reference electrode was placed in the rectum. After measurements started, time was allowed for the animal to equilibrate in air and a steady base line to be obtained. The platform was then placed in the Vickers Small Animal Chamber, through which oxygen passed at a flow rate of 4 l/min. After steady readings had been reached in 100% oxygen, the pressure was incrementally increased to a maximum of 45 lbfin^{-2} (310 kPa) absolute chamber pressure. Time was allowed at each pressure for measurements to reach equilibrium values. The tank was then decompressed to 100% oxygen at atmospheric pressure and the animal was removed and allowed to breathe air.

After the experiment the mouse was killed with an overdose of anaesthetic. With the electrodes fixed in position, the tumour was dissected from the animal. The positions of the electrodes were marked using ink (Pelikan, black) after dissection of the tumour. The position of the electrodes could be determined with some accuracy from histological sections when the tumour was fixed with the electrode *in situ*, and this was done in a few instances. The histological structure of the tumour was determined, and results from mammary adenocarcinoma only were included. 95% of the mammary tumours in the C_3H/Bts colony are adenocarcinoma [9].

6. *Assessment of skin damage*

The skin damage scale used is set out in Table 1. It does not differ much from that used by other workers in assessing damage to mouse skin in other sites [12], but is slightly easier to apply to pigmented C_3H skin over a tumour. All reactions were judged separately by two independent observers, and the mice were coded so that treatment details were not known to the observer. (This also applied to all tumour measurements.)

Table 1. *Skin reaction over tumour—(plucked skin)*

Very tiny skin flakes	$\frac{1}{4}$
Cracking in skin folds	$\frac{1}{2}$
Marked cracking	1
Scaling	2
Dry scab formed	3
Dampness	$3\frac{1}{2}$
Wet scab formed	4
Bad wet scab	$4\frac{1}{2}$
Ulcer	5

7. *Post-mortem examination*

All mice, control or treated, were examined *post-mortem*, and histological preparations made of all tumours, irradiated or not. If a mouse died before substantial post-irradiation tumour regrowth had occurred the irradiation site was examined for viable tumour cells.

The incidence of pulmonary metastatic deposits in these mice was recorded at *post mortem* examination, and extended with histological observations. If arterial emboli of mammary tumour cells were unequivocally visible on sections of lung tissue, the mouse was scored as positive for pulmonary metastatic deposits despite no gross lesion having been found at macroscopic examination.

RESULTS

(A) *A thousand rad in HPO combined with pretreatment with methotrexate* 7 days *before irradiation*

Groups of mice bearing tumours in the thoracic region only were allocated to four different treatment groups:

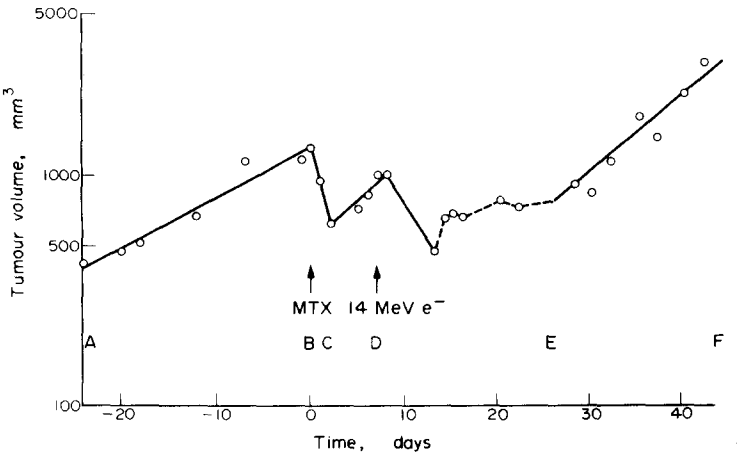


Fig. 1. Tumour volume changes in a spontaneous mouse mammary tumour. AB—pretreatment volume doubling time calculated (12.4 days). *At B,* 80 mg/kg *methotrexate injected into the tumour results in decreased tumour volume. C–D regrowth after methotrexate treatment—(volume doubling time* 9.0 days). *Response to methotrexate* = 0.36. *At D,* 1000 rad 14 MeV *electrons in HPO, with again decrease in tumour volume. D–E irregular volume changes due to oedema, etc. E–F regrowth after irradiation—volume doubling time* 8.8 days. *Response to* 1000 rad *HPO* = 0.72.

(1) 1000 rad in HPO (2) 80 mg/kg methotrexate intratumour + anaesthesia and HPO (3) 80 mg/kg methotrexate intratumour 7 days before 1000 rad in HPO (4) control mice subjected to anaesthesia and HPO treatment only.

As the supply of mice bearing mammary tumours on the thorax is about 4–8 mice/week from the C_3H/Bts colony, experimental groups of 8–12 mice at a time were accumulated over a period of 2–3 weeks. During this time the volume doubling times of the tumours were calculated, and treatment groups made up to contain equal numbers of faster and slower growing tumours. The range of volume doubling times of tumours used was 4.6–47.3 days, with a mean of 14.3 days. Tumours were irradiated at a relative volume of 1000–1200 mm^3. Exceptionally 24 mice at once were available for experiment, and altogether 56 mice were used in 4 experiments. One mouse from each treatment group was included in each group of 4 pressurized mice.

After injection of methotrexate into the tumour, the tumour stopped growing and decreased in volume. After 4–5 days growth started again, so that when irradiation was carried out 7 days after methotrexate injection the relative volume of the injected tumour had often but not always regained its pre-injection volume at the time of irradiation. (Figure 1 shows a typical growth curve for a tumour treated in this way, to illustrate the time relationships.) In this treatment group it is therefore possible to calculate the response of the same tumour first to methotrexate and then to 1000 rad HPO. It can be seen (Fig. 2a) that the tumour response of mice given methotrexate only, 0.22 ± 0.04, agrees well with the response, 0.23 ± 0.06, of the tumour in mice given methotrexate 7 days before irradiation. This response also agrees with the value of 0.20 ± 0.02 obtained from a larger group of 32 mice, neither anaesthetized nor subjected to HPO given this

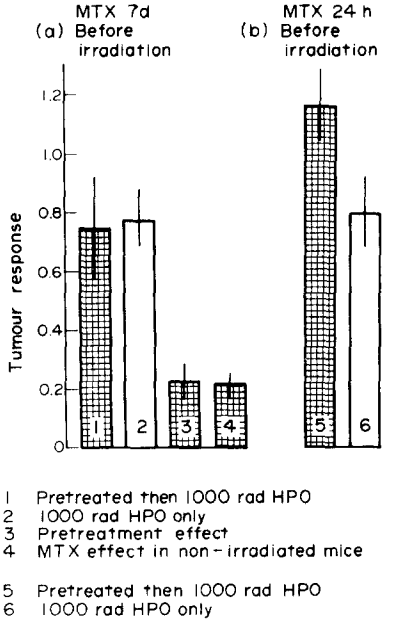


Fig. 2(a). Effect of 80 mg/kg *methotrexate given* 7 days *before* 1000 rad *in HPO.*

Fig. 2(b). Effect of 80 mg/kg *methotrexate given* 24 hr *before* 1000 rad *in HPO. Hatched blocks indicate methotrexate-treated tumours. Open blocks indicate tumour irradiated only. Vertical bars represent ± one S.E.M.*

dose of methotrexate in a dose–response assay [9]. Mice given 1000 rad HPO showed a tumour response of 0.78 ± 0.09, and this does not differ from the response to 1000 rad after methotrexate pretreatment, which was 0.75 ± 0.17.

The growth of tumours on mice subjected only to anaesthesia and compression was not affected.

(B) *A thousand rad in HPO combined with pretreatment with methotrexate* 24 hr *before irradiation*

A further 24 mice were used in 2 experiments where the interval between methotrexate pretreatment and 1000 rad HPO was reduced to 24 hr. As the response obtained to the standard dose of methotrexate was so reproducible the methotrexate only and procedural control groups were omitted. Each batch of 4 mice irradiated was made up of 2 pretreated and 2 non-pretreated mice. Figure 2(b) shows the result obtained. The mean tumour response to 1000 rad HPO, 0.80 ± 0.12, agrees well with the response to the same dose of irradiation, 0.78 ± 0.09, in the first 4 experiments. The response of the pretreated tumours is 1.16 ± 0.12 which is significantly greater than that of the non-pretreated tumours at the 0.05% level. The increase in response is approximately equivalent to the effect that 80 mg/kg methotrexate produces on its own. A combined methotrexate treatment and hyperbaric irradiation within a 24-hr period therefore produced an additive effect.

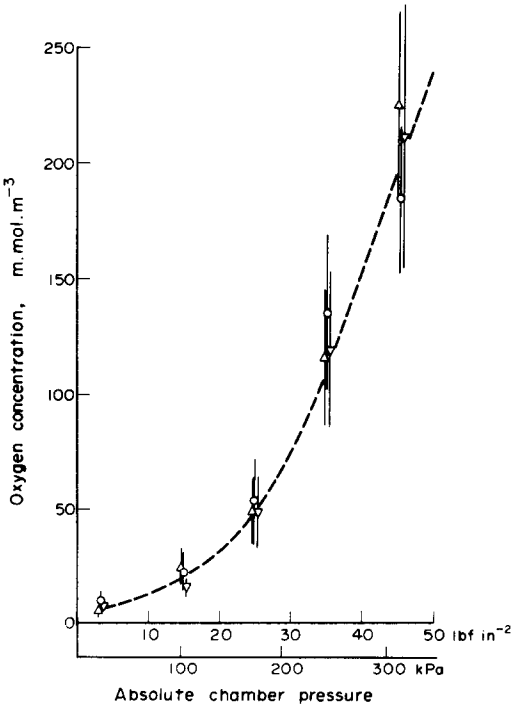


Fig. 3. Relationship between oxygen concentration in mmol m^{-3} *and absolute chamber pressure in* lbf in^{-2} *and* kPa *for mice treated with methotrexate* 24 hr *and* 7 days *before measurement and control, untreated mice. Circles represent mean values from untreated mice. Triangles represent* 24 hr *pretreatment, and inverted triangles* 7 day *pretreatment. Vertical bars represent* $\pm$ *one S.E.M.*

(C) *Oxygen concentration measurements in tumours*

The mice were divided into three groups to mimic the time intervals used in the irradiation experiment, i.e. (i) tumours pretreated with methotrexate 24 hr before oxygen measurements. (ii) tumours treated with methotrexate 7 days before measurement. (iii) untreated control group.

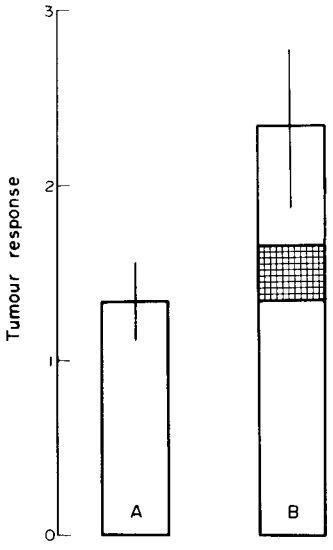
Figure 3 shows the relationship between oxygen cathode readings expressed as oxygen concentration in m mole m^{-3} against absolute chamber pressure lbf in^{-2} and kPa. The same line may be fitted to the mean response of tumours from untreated mice, and mice treated with methotrexate either 24 hr or 7 days beforehand, showing that there was no essential difference in rate or degree of oxygenation between the three treatment groups.

(D) *Three thousand rad in HPO combined with methotrexate given after irradiation*

In the remaining 3 experiments 32 mice were used. Skin over the tumours was plucked 24 hr before irradiation. All mice were irradiated with 3000 rad in HPO to the tumour, and 7 days later 16 mice were given 80 mg/kg methotrexate into the tumour. This group was then given citrovorum 200 mg/kg 24 hr after methotrexate. Both tumour response and the response to irradiation of the epilated skin over the tumour were assessed.

Tumour response

During the 300 day period of follow-up after treatment, 6 mice, 3 in each group, died or were killed before the treated tumour started to regrow. In three cases no cells recognizable as viable tumour cells were found in histological examination of the irradiation site, but in all cases it was assumed that regrowth would have taken place at the pretreatment rate starting the day after death, and regrowth curves were constructed accordingly. Figure 4 shows the mean response of the tumours to 3000 rad in HPO, and to 3000 rad in HPO followed by the standard dose of methotrexate (1.43 ± 0.21 and 2.43 ± 0.44 respectively). The hatched portion of the histogram indicates the contribution made by this dose of methotrexate injected into a tumour that has not been irradiated, and it can be seen that an additive effect is not, here, enough to account for the enhanced response.



A 3000 rad HPO
B 3000 rad HPO + MTX

Fig. 4. Tumour response to 3000 rad *in HPO with or without* 80 mg/kg *methotrexate given* 7 days *after irradiation. The hatched portion on col B represents the mean tumour response to* 80 mg/kg *methotrexate in non-irradiated tumours. Vertical bars represent* ± *one S.E.M.*

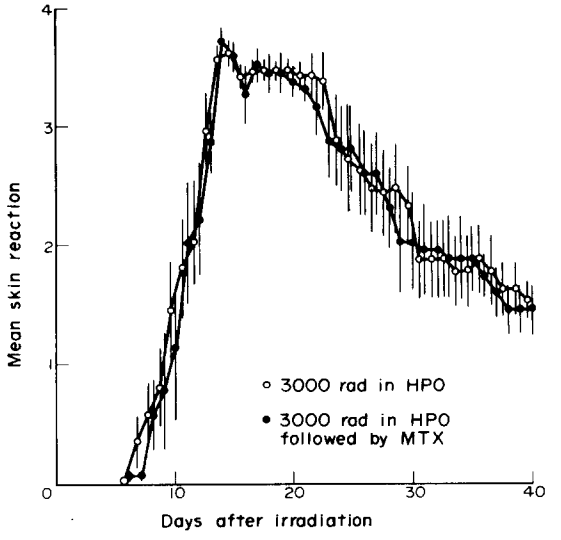


Fig. 5. Skin response to 3000 rad *in HPO, alone or followed by methotrexate administration. Vertical bars represent* ± *one S.E.M.*

Skin response

(a) *Early skin reactions.* Since methotrexate is not a specific "anti-tumour" agent, but will block cell division in the basal layer of the epidermis also, it would not have been altogether surprising if the skin reaction to 3000 rad in HPO had been more severe, or had followed a different time course, in mice which were given 80 mg/kg methotrexate intratumour 7 days after irradiation. The mean 6–30 day skin reaction was 2.4 ± 0.46 in the combined treatment group and 2.3 ± 0.41 in the irradiated only group. Figure 5 shows that the time of onset of skin damage was the same in both groups, and that the rate of repair to 40 days was substantially the same. The injection of methotrexate at a time after irradiation, just before irradiation-induced skin damage became apparent, did not delay healing.

(b) *Late skin reactions.* These were recorded in the 16 mice surviving more than 50 days after irradiation, 8 from each treatment group. Six out of 8 mice (75%) given methotrexate and irradiation showed some form of late damage at the irradiation site—2 out of 8 developed papillomas, in one mouse the skin became scarred and fibrosed and in 3 more there was late skin breakdown at 92, 114 and 128 days after irradiation respectively. Mice given irradiation alone showed the same incidence of papilloma formation, 2 out of 8, but only one late skin breakdown, at 98 days. Since these numbers are so small it is not possible to draw any firm conclusion from them.

Survival and lung metastases in all experiments

Survival data in these experiments are not clearcut, as it was necessary to kill 24 mice out of 112 because secondary mammary tumours were causing what was judged to be excessive discomfort to the mouse, including bleeding or ulceration of the tumour or obstruction of the anus. Despite this, certain points may be made. Table 2 shows the mean survival times after 1000 rad and 3000 rad for the different combinations of treatment and for methotrexate treatment alone. Mice given combined methotrexate and 1000 rad showed no increased survival compared to those given 1000 rad only, but the combined treatment had a slight beneficial effect on survival after 3000 rad. Survival after 3000 rad to the tumour, as might be expected, was longer than after the lower dose of irradiation.

The tumours of both methotrexate-treated and non-methotrexate-induced mice would be expected to suffer a certain degree of mechanical disruption following injection, with a concomitant increase in the incidence of pulmonary metastatic deposits [13]. The usual incidence in untreated C_3H/Bts mice is around 30%, and the incidence in combined and 1000 HPO irradiation only treated groups in these experiments was 39 and 45% respectively, increasing to 60% in both 3000 rad groups in which survival was longer. There is therefore no evidence that methotrexate administration altered the degree of metastatic spread from the treated tumours.

Table 2. Mean survival time after treatment

		Days ± S.E.
experiments 1–4	No treatment	43.1 ± 9.8
	Methotrexate 80 mg/kg	48.7 ± 7.3
	1000 rad HPO	64.5 ± 9.5
	1000 rad + methotrexate 7 days earlier	62.5 ± 10.5
5–6	1000 rad HPO	74.1 ± 4.1
	1000 rad + methotrexate 24 hr earlier	72.6 ± 12.2
	3000 rad HPO	105.2 ± 15.3
	3000 rad + methotrexate 7 days later	139.0 ± 17.4

DISCUSSION

The combination of methotrexate pretreatment and radiation has been claimed to be more efficacious than radiation alone, although clinical trials of this point present at the moment conflicting evidence. In the present animal experiments, a comparison was made between hyperbaric irradiation alone and hyperbaric irradiation combined with a single large dose of methotrexate at different time intervals. The spontaneous mammary tumour of the C_3H mouse is a relatively well oxygenated tumour, with a hypoxic cell population of perhaps 1% [14]. Nonetheless, irradiation under hyperbaric oxygenation produces a better tumour response to irradiation than under eubaric aerobic conditions, and it was decided to use hyperbaric irradiation in order to obtain the most favourable conditions for radiation damage. (Methotrexate therapy is used by some radiotherapists routinely in combination with hyperbaric irradiation.)

The administration of methotrexate 7 days before irradiation had no effect on the response of the tumour to 1000 rad. When the interval between methotrexate pretreatment and 1000 rad was shortened to 24 hr, a significantly increased radiation response was obtained. The amount of increase in response was such that we consider that this was essentially an additive effect of two submaximal killing agents upon the tumour cells.

Since the 1000 rad doses were given under hyperbaric conditions, it is theoretically possible that any increase in radiation response after methotrexate treatment might have resulted from increased effectiveness of tumour oxygenation under HPO. For example, loss of tumour volume might have allowed better distribution of oxygen to hypoxic cells, or damaged cells might have exhibited reduced oxygen utilization. The fact that we observed no difference in response to hyperbaric oxygenation for untreated tumours, tumours 7 days after methotrexate and tumours 24 hr after methotrexate indicates that the increased radiation response of tumours in the 24 hr pretreatment group is not caused by alterations in tumour oxygenation.

It is also possible that the modes of action of methotrexate and radiation may be independent of each other. If different targets are damaged by the two agents, then when radiation is given 7 days after methotrexate, repopulation of methotrexate-killed cells may have occurred, with the result that overall tumour response does not increase as it does with an interval of only 24 hr between the two agents. Friedman, de Narvaes and Daly [15] believe that methotrexate and radiation have their own specific modality in affecting tumour cells, in that methotrexate given before irradiation does not specifically enhance the effect of irradiation on the cells.

Berry, on the other hand, suggested that methotrexate might act by potentiating the effect of radiation upon the anoxic or hypoxic cells in a tumour more than on the well-oxygenated cells of normal tissues; he observed a reduction in OER from 2.6 to 1.8 for tumour cells surviving high drug doses [16].

The increased efficacy of methotrexate given 7 days after the higher dose of irradiation is believed to be more than additive, although, of course, synergistic effects cannot be proved at only one dose point. A possible explanation for this increase may be that phase-specific methotrexate may be more effective when irradiation has reduced the degree of asynchrony among the tumour cell population.

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μ-Chain Secretion by Peripheral Blood Lymphocytes in μ-Chain Disease; Functional and Ultrastructural Characterization of Cells Associated with μ-Chain Production*

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Abstract—*The course of secretion of immunoglobulin (Ig) μ-chain secretion by cultured peripheral blood lymphocytes in a case of heavy chain disease has been studied. It has been found that μ-chain protein is secreted primarily as a monomer. Dimers and polymers of μ-chain protein found in the serum of this patient are of secondary i.e. post-synthetic origin. By comparison of cytoplasmic and culture supernatant protein it has been found that metabolically inert deposits of μ-chain protein in aggregated form are present within the cultured cells. By ultrastructure studies these cells were classified as small lymphoid cells. The cells associated with μ-chain secretion in this patient exhibit distinct functional and ultrastructural features and possibly are arrested at a differentiation stage at which surface Ig synthesis is initiated.*

INTRODUCTION

BIOCHEMICAL studies with μ-chain protein (μCP) from three patients have shown that μCP in its monomeric form has a mol. wt in the range of 56,000 daltons [1–4] resp. 35,000 daltons [5]. All these patients had μCP of polymer type in the serum, showing the strong tendency of these abnormal proteins to aggregate.

By analysis of the cell sap of a μ-chain secreting tumor molecules of a mol. wt in the range of 10,000–350,000 have been observed [6] and it has been proposed on the grounds of ultrastructure studies that μCP is released by a process of limited cytolysis from enlarged cytoplasmic sacs or vacuoles visible in the cells [7].

We observed a 52 yr old female patient (Be.) with μCD. The finding of a high percentage of PBL staining positive for surface Ig of μ chain specificity and regrowing of these membrane-bound molecules after trypsination prompted us to perform short time cultures of PBL of this patient. My chains could be found regularly in the supernatant of such cultures. We used this approach to answer the following questions: 1. Is the μCP detected in the serum of patient Be. of heterogeneous type and what is the size of the monomer? If heterogeneous, i.e. if polymeric μCP is present, a second question should be answered. 2. Is this type of heterogeneity also found in culture supernatant of PBL of this patient? If μCP of high mol. wt is found in culture supernatant a third question has to be answered. 3. Does the comparison of cytoplasmic and secreted internally labelled μCP provide an answer to the question if synthesis and secretion of μCP of different mol. wt show a different time course?

MATERIAL AND METHODS

The diagnosis of μCD has been made in a 52 yr old female patient on the grounds of an Ig μ-chain related protein which has been detected by a combination method of starch gel electrophoresis and immunodiffusion [8]. Main symptoms of the patient were generalized lymphadenopathy, enlargement of the liver and the spleen and hemorrhagic complications. Details of this case are reported elsewhere [9]. Isolation of PBL was performed by density gradient centrifugation [10] in Ficoll–Isopaque (Lymphoprep®, Nyegaard & Co., Oslo). Venous blood was drawn in heparin (Liquemin®, Roche) 0.4 ml/20/ml blood. For culture the cells were washed three times in HBSS.

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Immunoelectrophoresis [11] and Ouchterlony analyses have been done using anti human serum from the rabbit (Medac/Hamburg) and μ-chain specific antisera (Behring-Werke AG, Marburg) in agar pur. (Behring-Werke AG./Marburg). Isolation of μCP has been performed by immunoadsorption on polyacrylamide particles [12] coated with μ-chain antibodies (Medac/Hamburg). SDS–PAGE [13, 14] has been done in a Multiphor apparatus (LKB-Producter AB/Bromma, Sweden) using Combithek® (Boehringer/Mannheim), an Ig light chain protein of kappa type and a reduced alkylated macroglobulin of Waldenström type as marker proteins. Densitometric tracings of gels after staining with Coomassie Brilliant Blue (0.05%) were done in the DD2 apparatus (Kipp and Zonen, Delft/Netherlands).

L-[U-^{14}C] leucine (324 mCi/mmol, RadioAmersham, G.B.) has been used for radiolabelling of μCP in cell cultures. Radioactivity in gels has been measured (Packard Tricarb.) using solubilizer TS-1 (Koch-Light Lab. LTD, G.B.) after dissection of the gel in slices of 1 mm thickness. Anti μ-chain antibodies (Medac/Hamburg) have been labelled with ^{125}I using the Bolton–Hunter reagent (NEN/Boston, U.S.A.). For the detection of μCP after gel electrophoresis Whatman 3 mm paper soaked with antiserum was layered on to the gel for 24 hr in a humid chamber. Radioactivity in slices of the gel was measured after washing the gel in saline for 24 hr.

Short time cultures have been done in TC Medium 199 (Difco Lab./Detroit, USA) with 50 $\times 10^6$ PBL/ml. A total of 250×10^6 cells was used and 10 μCi/ml of ^{14}C-leucine was added. Culture supernatant has been collected at various days of culture and dialyzed exhaustively against PBS. For the study of the time course of secretion of μCP 4×10^6 cell/ml were used. A total of 40 $\times 10^6$ cell in the presence of 5 μCi/ml ^{14}C-leucine were used.

For reduction and alkylation of μCP and IgM protein 0.75 m mercaptoethanol [15] (Merck/Darmstadt) and iodoacetamide (Merch/Darmstadt) or dithiotreitol [5] (Calbiochem, San Diego, USA) and iodoacetamide has been used.

For SDS–PAGE cytoplasmic proteins of cultured cells (day 5 of culture) after ultrasonication (Biosonik III, Bronwill Scientific, Rochester, U.S.A.) were prepared by centrifugation (18,000 ***g***, 10 min) in PBS.

For electron microscopy PBL together with other mononuclear leucocytes and platelets were isolated [10], washed three times in SBSS, fixed in suspension with 2% glutaraldehyde (in 1/15 m Sørensen buffer, pH 7.4, 2.55 mM $MgCl_2$, 500 mOSM), the isolation and fixation were carried out between 0° and 4°C; pellets of fixed cells were postfixed in 1% OsO_4 and 1% uranyl acetate solution, embedded in Epon 812, cut into ultrathin sections with a LKB Ultratom and examined with a Philips EM 300 electron microscope at 60 kV.

RESULTS

Figure 1 shows the result of SDS–PAGE of isolated μCP from the serum of the patient Be. (A). Three components of 65,000, 84,000 and 220,000 daltons are visible. After reduction and alkylation using mercaptoethanol as reducing agent a main component of 38,000 daltons is observed (B). Smaller components of 18,000 and 65,000 daltons are present. Using dithiotreitol for reduction a main component of 42,000 daltons is observed; minor components of 66,000 and 93,000 daltons are present.

In an earlier experiment with mercaptoethanol 5 components of 16,000, 32,000, 69,000, 93,000 and 182,000 daltons have been found, the component of 32,000 daltons being the main fraction.

With supernatant of PBL cultured for 4 days (Fig. 1, C) four components of 18,000, 41,000, 65,000 and 142,000 daltons are observed in SDS–PAGE. Reduced and alkylated monoclonal IgM protein shows five components of 11,000, 20,000, 33,000, 65,000 and 95,000 daltons.

Radiolabelled protein after SDS–PAGE of culture supernatant collected at day 1, 3 and 5 and of cytoplasmic protein (CP) after ^{14}C-leucine incorporation is shown in Fig. 2. From the start of electrophoresis (left side) radioactivity decreases to a level of about 0.5×10^2 counts/min and rises to a major peak at 30,000 daltons and a minor peak at 12,000 daltons on day 1. On day 3 a zone of radiolabelled protein appears in the region of 20,000–68,000 daltons. On day 5 a sharp peak of 15×10^2 counts/min arises at 30,000 daltons.

Radiolabelled cytoplasmic proteins (CP) appear in a more heterogeneous distribution in a region of 20,000–68,000 daltons with maximum activity of about 100×10^2 counts/min. Localization of μCP within the gel is shown in Fig. 3. Again, the peak maximum of internally labelled protein is seen at a position of about 30,000 daltons (Fig. 3, upper part).

Binding of ^{125}I-labelled μ-chain antibodies (Fig. 3, lower part) obviously corresponds to the distribution of radiolabel with a peak at 30,000 daltons. A second minor peak of ^{14}C-leucine

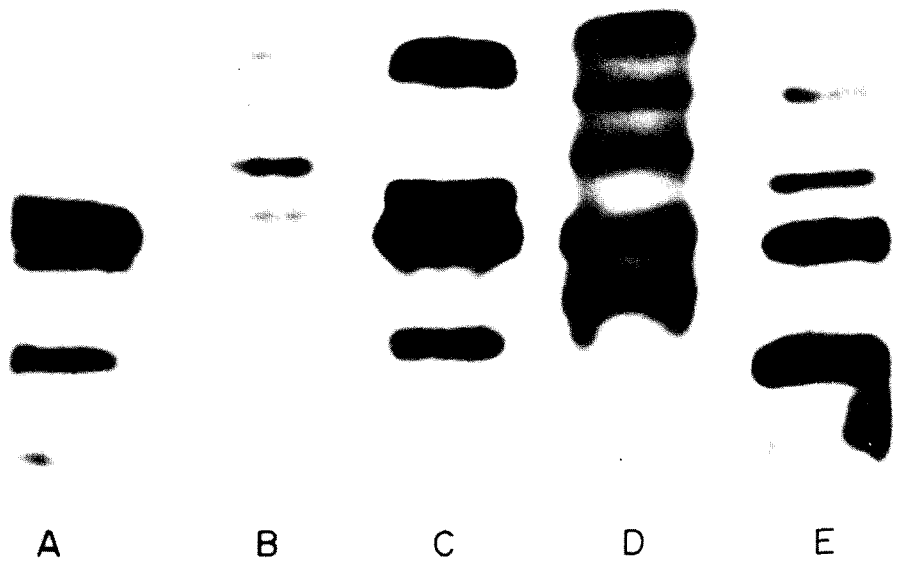

Fig. 1. Electrophoresis in SDS–PAGE of isolated μCP of patient Be. (A), *reduced and alkylated μCP* (B), *supernatant of a culture* (4 days) *of PBL of patient Be.* (C), *reduced and alkylated IgM protein* (D), *marker proteins* (E).

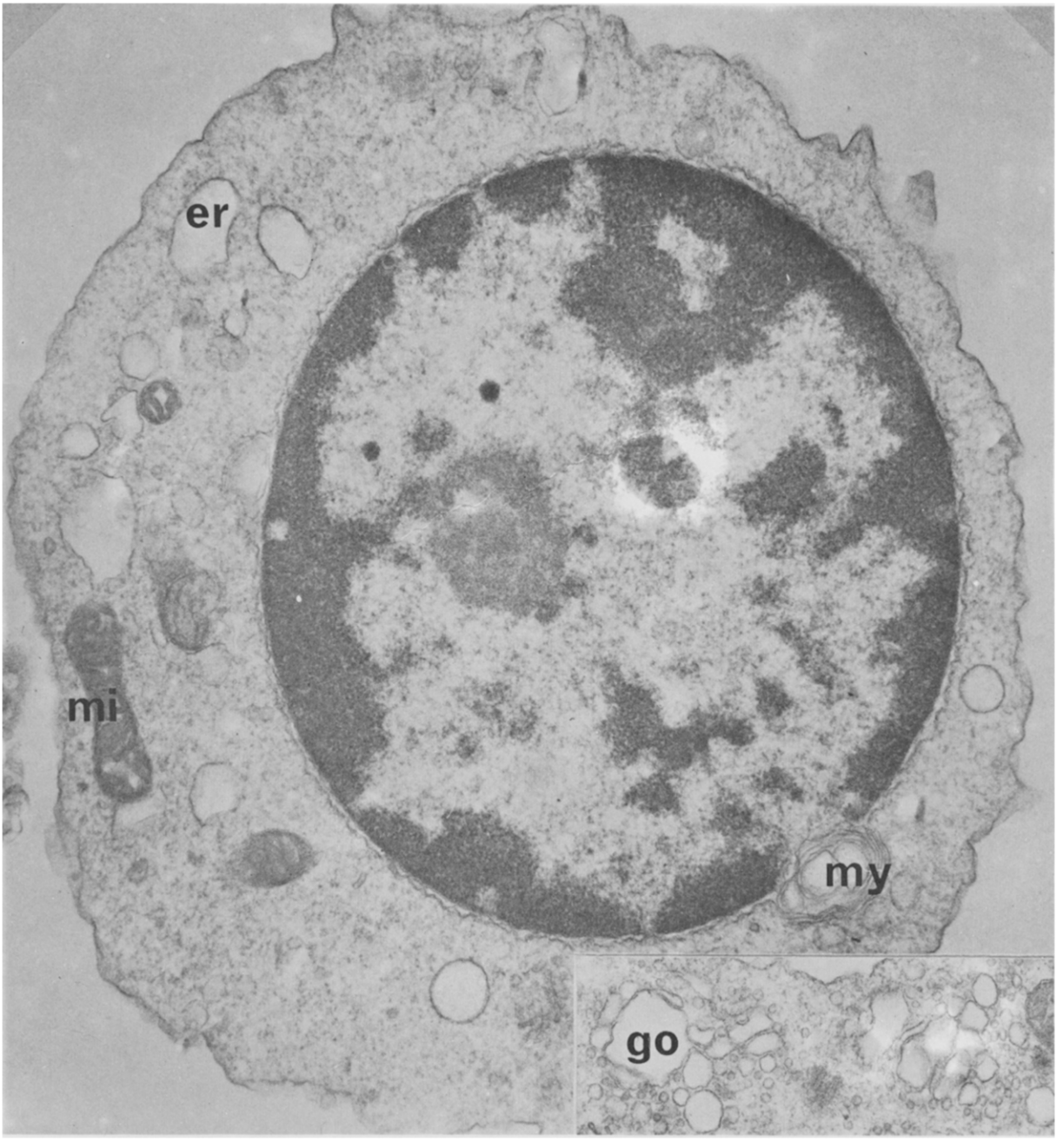

Fig. 5. Predominant type of lymphocyte in patients peripheral blood. Swollen cisternae of the endoplasmic reticulum (ER), swollen Golgi stacks (GO, inset showing section through Golgi zone) and mitochondria (MI) in the condensed state are the main characteristics of this cell type. Occasionally myelin figures (MY) were observed in the cytoplasm. Magnification × 40,000.

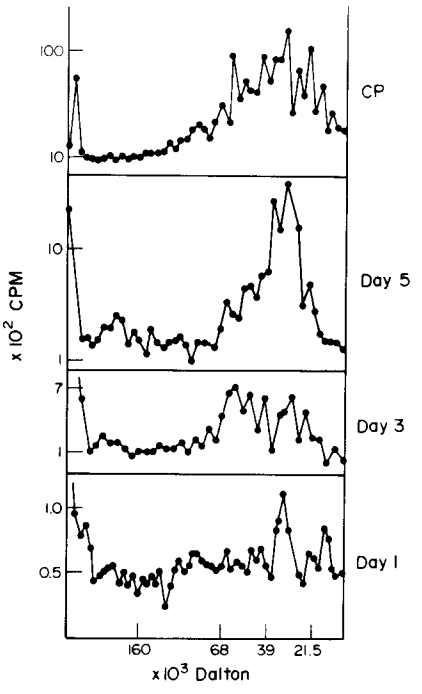

Fig. 2. SDS–PAGE of internally ^{14}C*-leucine labeled protein in supernatant of* day 1, 3, 5 *of culture of PBL of patient Be. and cytoplasmic protein (CP) at* day 5 *of culture.*

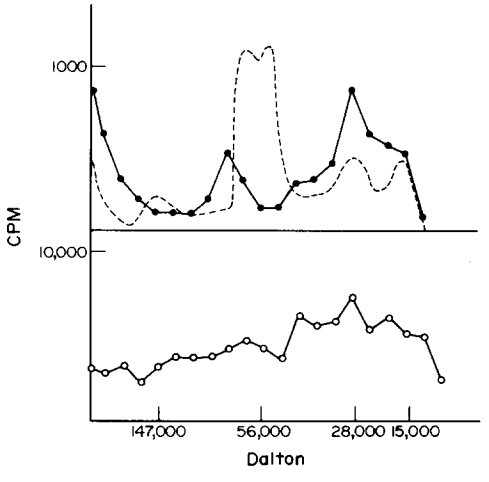

Fig. 3. SDS–PAGE of supernatant of a culture (4 days) *of PBL of patient Be. in the presence of* ^{14}C*-leucine.*
Upper part: densitometric tracing (——), ^{14}C*-leucine incorporation* (●——●).
Lower part: result of binding of ^{125}J *labelled μ chain antibodies* (○——○).

labelled protein corresponding to 78,000 daltons without significant binding of μ-chain antibodies has been observed in this experiment.

A comparison between the size distribution of internally radiolabelled cytoplasmic μCP and "cold" cytoplasmic μCP is shown in Fig. 4. Cytoplasmic protein of cultured PBL after a period of 5 days (from the experiment shown by Fig. 2) were used. The maximum binding (20 $\times 10^3$ counts/min) of ^{125}I-labelled antibodies (Fig. 4, lower part) is observed in a region of 160,000 and 110,000 daltons. In contrast to internally labelled μCP (Fig. 4, upper part) "cold" μCP resides at a position in the gel which corresponds to a significantly lower mol. wt. Virtually no ^{14}C-leucine radioactivity has been observed in the region of maximum binding of labelled μ-chain antibodies.

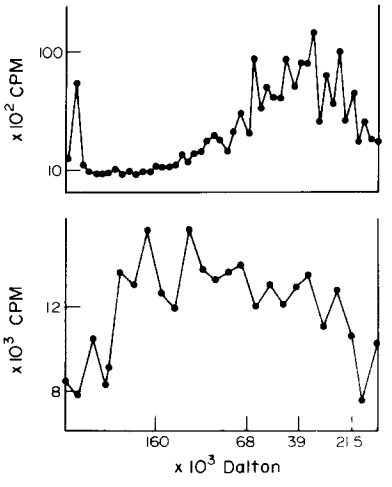

Fig. 4. Comparison of SDS–PAGE of cytoplasmic μCP after 5 days *of culture in presence of* ^{14}C*-leucine. Internally labelled μCP (upper part) and "cold" μCP (lower part).*

The results of SDS–PAGE of isolated serum μCP, reduced and alkylated serum μCP and culture supernatant μCP are shown in Table 1. Some variation of the numbers due to preparation and methodological hazards is obvious. It follows from these figures that culture supernatant proteins show a greater heterogeneity than μCP from the serum, especially in the region below 65,000 daltons. A strong "new" component of 372,000 resp. 355,000 daltons has been observed.

Figure 5 shows the result of ultrastructural analysis of PBL of the patient by transmission electron microscopy. The predominant cell type (80 out of 100 sections of cells studied) is a small lymphoid cell, showing some striking differences with the usual type of circulating small lymphocyte. First, whereas in normal PBL essentially no ER is present, patients PBL show a considerable number of smaller (smooth and rough) ER-cisternae; moreover, the Golgi stacks in these cells are invariably extended. Second, in contrast with the conformation of the mitochondria in normal PBL the mitochondria in patients PBL are all in the condensed state [16]. Third, the nucleocytoplasmic ratio of patients PBL appeared to be considerably lower than in normal PBL. No nuclear or nucleolar alterations were observed.

Table 1. Results of SDS–PAGE of μCP under various conditions. Main components as judged by densitometer tracings are underlined

Isolated serum μCP, untreated		Reduced and alkylated serum μCP			Culture supernatant μCP, untreated		
1	2	1*	2*	3†	Day 1	Day 3	Day 5
		18,000	16,000		17,000	18,000	15,000
					26,000	25,000	23,000
	38,000	38,000	32,000		32,000	33,000	
				42,000	48,000	51,000	51,000
65,000	69,000	65,000	69,000	66,000	63,000	66,000	62,000
84,000					83,000		
	93,000		93,000	93,000			90,000
					93,400	105,000	
220,000	158,000		182,000				
					372,000	355,000	

*Reduction by mercaptoethanol
†Reduction by dithiotreitol

DISCUSSION

Heterogeneity of μCP has been shown to result from intracellular synthesis in the first and hitherto only report [6] on biosynthesis of μCP. It has been also shown by these authors [6] that almost all of the secreted μCP is of a greater size than 350,000 daltons. In contrast to this observation the majority of μCP secreted by cultured PBL of the patient Be. described here is of considerably smaller size and is of the same order of size as the monomer of serum μCP (about 40,000 daltons). Cultured PBL of the patient Be. secrete μCP of low mol. wt which has not been found in the serum using immunoadsorption on antibody coated polyacrylamide beads. It seems possible, therefore, that polymer type μCP results in part from the isolation procedure as it has been discussed by the above cited authors [1]. Another factor, which may play a role as to electrophoretic behavior is storage of μCP. We observed changes of the pattern of μCP components in SDS–PAGE occurring after 4 and 7 days storage at 4°C with μCP of culture supernatant (unpublished results). From the comparison of the mol. wt of secreted μCP and cytoplasmic μCP (Fig. 4) follows that the major part of cytoplasmic μCP of a higher mol. wt than 100,000 daltons remains unlabelled after 5 days of culture in the presence of ^{14}C-leucine. The internally radiolabelled cytoplasmic μCP shows the same mol. wt as the secreted μCP. This finding and the time course of secretion (Fig. 2) clearly indicates that monomer is the form of μCP which is primarily synthesized and does not originate from intracellular or extracellular degradation. From the fact that a large amount of μCP remains unlabelled within the cells and virtually no radiolabelled molecules of more than 70,000 daltons are released into supernatant we conclude that μCP of higher mol. wt cannot be exteriorized properly by the cells and form a sort of metabolically inert deposits within the cell. The mechanism by which these aggregates are discharged by the cells is unknown. In order to evaluate the possibility that this material originates from cell disintegration the viability of cultured cells and radioactivity in the supernatant after ^{14}C-leucine incorporation over a period of 13 days has been measured. A strong resemblance of the course of cell number and supernatant radioactivity has been observed indicating that labelled protein is the product of functional intact cells (unpublished results). It has been suggested on the grounds of ultrastructure studies that μCP is released by a process of limited cytolysis from vacuoles visible in bone marrow cells of plasma cell type of a patient with μCD [7]. The presence of numerous cytoplasmic vacuoles is a common ultrastructural feature of the cells of the above mentioned case [7] and our patient. Cytoplasmic vacuoles of plasma cells have been found in another case [17] of μCD by light microscopy. These vacuoles have not been found in cells associated with the production of free γ or α heavy chains or free light chains [18]. Considering the obvious ultrastructural differences between the two types of μCP producing cells discussed here the observation of these vacuoles may indicate a common basic defect and it can be speculated that the cells represent different maturation steps of the lymphoplasmacytoid B-series. The fact that in our case small lymphoid cells were associated with μCP production and that a significant percentage of these cells showed S.Ig of μ-chain specificity by immunofluorescence techniques [9] as well as the failure of mitogenic stimulation by PHA and

Con A and response by PWM [9] is compatible with this assumption. With respect to the origin of the cytoplasmic vacuoles of μCP producing cells it is of interest to note the swollen Golgi stacks (Fig. 5). A similar observation has been made by the cited authors [7]. This correspondence supports the view that the vacuoles originate from the Golgi complex. The presence of a minor component of 18,000 daltons after reduction and alkylation of μCP using mercaptoethanol (Fig. 1) has to be discussed. A component of about this mol. wt has been observed in the culture supernatant as well. Isolated μCP from the serum of the patient Be. and culture supernatant, therefore, has been tested for the presence of J chains which are of about this mol. wt [19, 20] and which have been found in a case of μCD [5]. By the analysis in Ouchterlony plates using an antiserum against J chains kindly supplied by Dr. Brandtzaeg/Oslo no precipitation line has been detected. Reduced and alkylated monoclonal IgM protein has been used as a control. The nature of this component remains obscure for the moment. The fact that a component of this mol. wt has not been seen using dithiotreitol for reduction of μCP does not support the view that it represents the monomer unit of μCP.

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Demonstration of a Wilms' Tumour Associated Antigen Using Xenogenic Antiserum (Preliminary Communication)*

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Abstract—*In this report we describe the preparation and partial characterization of a mono-specific anti-Wilms' antiserum raised in rabbits which had previously been injected normal kidney extracts* in utero. *The tumour associated antigen in Wilms' tumour does not appear to be oncofoetal antigen and it has also failed to react with feutin. It is possible that both our antiserum and that of Burtin and Gendron may be directed against a similar cell component.*

INTRODUCTION

WILMS' tumour, a primary neoplasm of the kidney, is one of the common malignancies of early childhood [1]. Immune responses against tumour associated antigens (TAA) of Wilms' tumour have been investigated by a variety of techniques. Lymphocyte-recognised antigens have been demonstrated by tumour cell killing techniques [2–5] and cytoplasmic and membrane antigens by immunofluorescence [6, 7]. Other evidence for the presence of tumour associated substance in Wilms' tumour is provided by the work of Allerton *et al.* and Wise *et al.* who demonstrated an abnormal component in the serum, urine and tumour extracts of these patients [8, 9]. Xeno-antisera have also been used to demonstrate specific antigens in Wilms' tumour extracts [10]. In this report we describe the preparation and partial characterisation of a monospecific anti-Wilms' antiserum.

MATERIAL AND METHODS

Tissues were either fresh surgical biopsies or those stored in a liquid nitrogen bank [11].

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(A) *Tissue homogenates*

The kidney extracts used to tolerize rabbit foetuses (see below) were obtained by homogenizing aliquots of tissue fragments (10% w/v; Silverson Homogenizer), from three normal kidneys in 0.1 M phosphate buffered saline (pH 7.2) for 2 min at 0–4°C.

Tissue extracts used for raising the antiserum, immunochemical and absorption studies were obtained by homogenizing tissue (10% w/v) in EDTA by the method of Allerton *et al.* [8]. Protein estimations were carried out according to Lowry *et al.* [12].

(B) *Antiserum*

Attempts to tolerize the rabbits which were to be used for raising the antiserum against Wilms' tumour were made by injecting 2 week old foetuses *in utero* with 0.2 ml of normal kidney extract. These rabbits (4 litter mates) when 6 months old were inoculated intradermally with 0.5 ml of "pooled" EDTA extract of 6 Wilms' tumours. Six injections without adjuvant were given at fortnightly intervals and a week after the last injection the rabbits were bled and the sera separated and pooled.

(C) *Tissue culture*

Cell cultures were established in a similar way to that described in our previous study [5]. Briefly, blood clot and necrosed areas were removed from the tissue fragments which were washed in medium 199, and macerated with scalpels and scissors. The resulting suspension

was grown either in roller bottles or in tissue culture flasks (Falcon Plastic). Cultures were usually confluent within 2 weeks of initiation. Morphologically two different types of cells were seen in culture: (a) fusiforms which resemble fibroblasts and (b) polygonal cells (25–35 μm in diameter) lying either by themselves or in epithelial-like sheets. Because the cells from a Wilms' tumour have a certain mesenchymal appearance in their nuclear and cytoplasmic pattern it is impossible to distinguish with certainty the stromal from neoplastic elements. However, there is a considerable variation in nuclear size suggesting that the outgrowth did contain neoplastic cells. Cells were harvested by a brief treatment with trypsin (0.25% Difco) and were either grown as a monolayer on glass coverslips in the wells of Leighton tubes for immunofluorescence or were homogenized for gel diffusion, absorption studies etc.

(D) *Gel diffusion and immunoelectrophoresis*

Gel diffusion plates were prepared by using 1% agar (Difco, Noble agar) in 0.85% saline. Immunoelectrophoresis was carried out at 4°C using 1% agarose in 0.05 M Barbitone buffer at pH 8.2 [13].

(E) *Immunofluorescence ("IF")*

Tissue cultured cells and cryostat sections were fixed in acetone and the indirect "IF" technique was carried out as described previously [6].

(F) *Absorptions*

The pooled antiserum was successively absorbed with freeze dried extracts (100–200 mg/ml) of pooled foetal kidney (12, 25, 31 and 37 weeks), neonatal and adult kidneys, liver, thymus, spleen, tissue cultured Chang cells, neuroblastoma, mammary carcinoma and pooled human sera. Absorptions with each of these absorbants was carried out individually at 4°C for 24 hr. After each absorption the precipitate was removed by centrifugation at 30,000 rev/min for 1 hr at 4°C.

RESULTS

The antiserum in gel diffusion produced several precipitation lines against extracts of Wilms' tumour, hypernephroma and kidneys obtained from 4 foetuses (12, 25, 31 and 37 weeks), 2 neonates (4 and 8 weeks), 2 adult and 5 normal-looking kidneys from Wilms' patients which were attached to the tumour mass but had no gross or microscopic evidence of tumour infiltration (Fig. 1a). The number of precipitation lines were the same against all ages of kidney and a reaction of complete identity was observed in all of them. The presence of an extra line against Wilms' tumour extract (Fig. 1a) indicated that although we may have succeeded in obtaining antibody against TAA, obviously our attempt to tolerize rabbits to normal renal antigens *in utero* were not successful. Therefore the pooled antiserum was absorbed with several tissue extracts, normal human serum etc. (see Methods). The absorbed antiserum produced a single precipitation line in gel diffusion against 4 Wilms' tumours (Fig. 1b and Table 1). An extract of hypernephroma also reacted with the antiserum while its tissue culture homogenate failed to produce any precipitation line. A complete reaction of identity was observed between extracts of Wilms' tumours, Wilms' tumour cell lines and hypernephroma. Control extract of normal human kidney, non-renal solid tumours including their cell cultures, serum samples from normal donors and patients with Wilms' tumour did not produce any precipitation line (Table 1).

Immunoelectrophoresis of Wilms' tumour extract against absorbed antiserum showed a single anodic band with α-mobility (Fig. 2).

Indirect immunofluorescence using absorbed antiserum (1:20 dilution) produced a strong diffuse intra-cytoplasmic staining of 4 Wilms' tumour and cultured cells derived from 2 Wilms' tumours and 1 hypernephroma (Table 1 and Fig. 3). By using class specific Ig (Wellcome, Beckenham, Kent, England) the type of antibody involved was identified as IgG. Similarly treated 5 non-renal tumours, 4 foetal kidneys, 2 neonatal kidneys, 2 adult and 4 normal looking kidneys were negative.

DISCUSSION

The antigenic substance associated with Wilms' tumour has been demonstrated using anti-Wilms' serum raised in rabbits. The TAA detected in Wilms' tumour does not appear to be an onco-foetal antigen as the absorbed antibody failed to react (both in gel diffusion and immunoelectrophoresis) with foetal kidney and liver from 4 foetuses (12, 25, 31 and 37 weeks), thymus, α-foeteprotein, carcinoembryonic antigen. Similarly, cryostat sections of foetal kidney, liver and thymus when tested by indirect "IF" were not stained by the absorbed antiserum. It is realised that more stringent testing would be necessary before our antiserum's reaction against onco-foetal antigen can be completely excluded.

Unlike Wise *et al.* [11] we failed to detect any

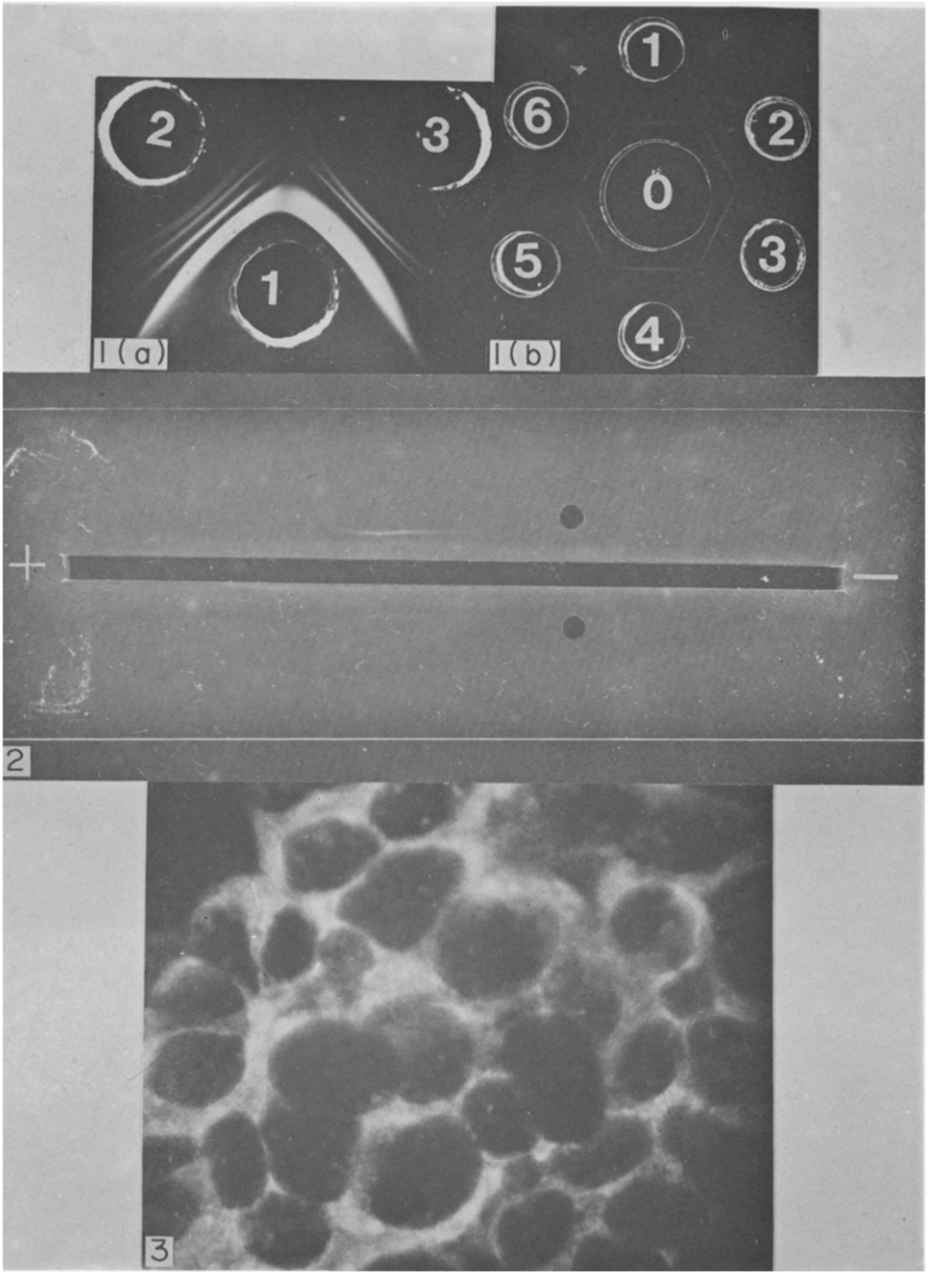

Fig. 1. (*a*). *Several precipitation lines were produced by unabsorbed antiserum* (*Well* 1) *against both Wilms' tumour* (*Well* 2) *and normal kidney* (*Well* 3) *extracts. Note the presence of an extra precipitation line between Well* 1 *and* 2.

Fig. 1. (*b*). *Gel diffusion studies using absorbed anti-Wilms' antiserum* (*Well* 0). *A reaction of identity can be seen between extracts of four Wilms' tumour* (*Well* 1, 2, 3 *and* 4), *hypernephroma* (*Well* 5) *and tissue cultured Wilms' tumour cells* (*Well* 6).

Fig. 2. *Immunoelectrophoretic pattern of Wilms' tumour extract* (*top and bottom wells*) *developed with purified IgG obtained from absorbed anti-Wilms' antiserum.*

Fig. 3. *Indirect immunofluorescence-localization of intracytoplasmic antigen in tissue cultured Wilms' tumour cells by the absorbed anti-Wilms' antiserum. The pattern is diffuse cytoplasmic* (*note the nuclei are not stained*).

Table 1. The results of gel-diffusion and immunofluorescence using absorbed anti-Wilms' antiserum

	Source	Gel diffusion	Immunofluorescence
(A)	Renal tumours		No. positive/No. tested
	(i) Wilms' tumour	4/5	4/4
	(ii) Hypernephroma	1/1	1/1
(B)	Non-renal solid tumours		
	(i) Neuroblastoma	0/2	0/2
	(ii) Breast carcinoma	0/2	0/2
	(iii) Glioma	0/2	0/1
(C)	Kidneys		
	(i) Foetal (12, 25, 31 and 37 weeks)	0/4	0/4
	(ii) Neonatal (4 and 8 weeks)	0/2	0/2
	(iii) Adult	0/2	0/2
	(iv) "Normal looking" kidney from Wilms' patients	0/5	0/4
(D)	Tissue culture cells derived from		
	(i) Wilms' tumour	2/6	2/2
	(ii) Hypernephroma	0/1	1/1
	(iii) Ewing's tumour	0/1	0/1
	(iv) Neuroblastoma	0/2	0/2
	(v) Breast carcinoma	0/1	0/1
	(vi) Chang cells	0/1	0/1
	(vii) Endothelial cell lines (human)	0/2	0/2
	(viii) Endothelial cell line (rat)	0/1	0/1
(E)	Serum samples		
	(i) Wilms' patient	0/2	0/3
	(ii) Normal serum	0/4	0/4

cross-reactivity of Wilms' antigen with fetuin present in foetal and newborn calf serum, using geldiffusion and immunoelectrophoresis. Absorption of our antiserum with foetal calf serum did not influence its "IF" staining of cryostat sections of Wilms' tumours. Thus far we have not absorbed the antiserum with purified fetuin. It is possible that both our antiserum and that of Burtin and Gendron [10] may be directed against a similar cell component as both antigens had the similar electrophoretic mobility. Both antisera also cross-reacted with hypernephroma. The presence of a common antigenic determinant in Wilms' tumour and hypernephroma supports our earlier lymphocytotoxicity results [5]. The antigen shared by the two tumour types was not a normal renal antigen as the antiserum neither produced immunofluorescence nor a precipitation line in gel diffusion against normal kidneys. We are very grateful to Dr. P. Burtin who has recently tested our antiserum and has confirmed its specific reactivity against Wilms' tumour extracts by gel diffusion and immunoelectrophoresis. He also found that the antiserum did not react with normal kidney.

Further studies are in progress to characterize TAA in Wilms' tumour extracts.

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Tumor-Related Blocking of Anti-Fetal Immunity*

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Abstract—*A modified spleen colony assay was employed to evaluate the effect of various tumor-related factors upon the* in vivo *effectiveness of anti-fetal immunity. Reduction of fetal liver (hemopoietic) colony formation by* in vitro *incubation of fetal liver cells with lymph node cells sensitized to syngeneic fetal liver or plasma cell tumor was blocked by* (1) *solubilized fetal antigen,* (2) *serum from mice recently immunized with syngeneic fetal liver and* (3) *serum from patients with metastatic colon cancer. In the latter, the degree of blocking correlated with plasma C.E.A levels.*

Both humoral and cell-mediated mechanisms of anti-fetal immunity were blocked in plasma cell tumor-bearing mice, suggesting in vivo *suppression of the immune response via circulating tumor-associated fetal antigen.*

INTRODUCTION

IMMUNIZATION with fetal cells confers modest protection against subsequent tumor challenge in some [1–6], but not all [7–13], animal systems. Conversely, immunization with methylcholanthrene (MCA)-induced tumor cells effected the resorption of developing mouse fetuses in a subsequent pregnancy [14], and the administration of antiserum to alpha-fetoprotein during pregnancy has caused abortion [15] or developmental abnormalities [16] in mice and rats. Castro *et al.* [11] have shown that embryoma development was more successful in thymectomized than in normal syngeneic recipients. Furthermore, the development of mouse fetal liver (hemopoietic) colonies in the spleens of irradiated syngeneic mouse recipients can be suppressed by preimmunization with various animal [17–19] or human [20, 21] tumor cells, including malignant melanoma, colon carcinoma, and myelogenous or lymphocytic leukemia. The evidence generated from such studies suggests that tumor-associated fetal antigens may indeed elicit transplantation-type immunity, and that they could play a critical role in tumor-host interactions.

Numerous factors have been suggested to account for the failure to obtain tumor protection via fetal immunity in some systems. Coggin and Anderson [22] have delineated several requirements for a successful fetal vaccine in the SV40 hamster tumor. Girardi *et al.* [3] correlated the parity status of hamsters with the appearance of serum blocking factors that can abrogate the cytotoxicity of lymphocytes from tumor-bearing animals against SV40 transformed cells. The Hellströms have noted the blocking activity of serum from pregnant or MCA-tumor-bearing (TB) mice in a microcytotoxicity assay of lymph node cells (LNC) from multiparous (MP) mice on cultivated syngeneic tumor cells [23] and unblocking by a rabbit antiserum to BALB/c embryonic tissues [24]. Studies with solubilized embryonic antigens and their corresponding antisera have suggested that only antigen–antibody complexes block efficiently at the target-cell level [25]. Baldwin *et al.* have also noted MP and TB serum blocking of the effects of MP-LNC cytotoxicity on a variety of chemically induced rat tumors. However, these investigators emphasized that MP serum could not block the tumor-specific cytotoxicity of TB-LNC [26]. Similarly, this group showed that blocking at the effector level with solubilized tumor-associated fetal antigen was only successful when MP-LNC, rather than TB-LNC were employed against cultured tumor target cells [27].

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These findings reflect the multiplicity of tumor-associated neoantigens that are susceptible to immunologic attack. Therefore, in attempting to define factors responsible for the failure of anti-fetal immunity, we have sought to reduce the complexity of the situation by avoiding the use of tumor cells as targets. Rather, we have examined the effect of various factors from the tumor setting in an *in vivo* assay of anti-fetal immunity.

MATERIAL AND METHODS

Colony-forming cells

Single cell suspensions were prepared from the liver of 15-day BALB/c fetuses (from primipara) and from adult femoral bone marrow by several passages of the tissue through an 18-gauge needle with Hank's balanced salt solution (HBSS).

Lymph node cells

Sensitized lymph node cells (LNC) were collected from mice which had been immunized with syngeneic 15-day fetal liver or plasma cell tumor (PCT). Mice were immunized 5 times i.p. over a 2 week period with 10^7 viable, irradiated cells (5000 rad ^{137}Cs). Non-immunized controls were injected with HBSS. LNC were collected one week after the last immunization.

The BALB/c PCT, originally induced with mineral oil, was passaged in males in ascites form. Fetal immunization has been shown to offer weak protection against this tumor [2]. An inoculum of 10^6 cells kills the host in approximately 2 weeks. LNC from tumor bearers were collected at day 7 or day 14.

The axial, brachial, inguinal and mesenteric lymph nodes were dissected aseptically and disrupted in a ground glass tissue homogenizer with HBSS. Single cells were recovered after passage through a 50-mesh screen and diluted to an appropriate concentration without further washes, or washed 6 times with HBSS.

Sera and antigens

Antigen was solubilized from BALB/c fetal or adult liver cells with 3M KCl by the procedure of Reisfeld *et al.* [28]. The extracts were stored at −70°C.

Serum was obtained from individual colonic carcinoma patients or pooled from normal male blood donors. Plasma CEA levels were determined by radioimmunoassay using zirconyl phosphate gel [29]. Human sera were heat-inactivated (56°C for 30 min) and absorbed *in vivo* (0.4 ml injected i.v. into 15–20 g male mice and collected by exsanguination 2 hr later) or *in vitro* v/v against normal BALB/c tissue (heart, lung, kidney, spleen and liver) for 1 hr at 37°C and overnight at 4°C.

Spleen-colony assay

The assay for development of fetal colony-forming units (FCFU) has been described in detail elsewhere [30, 17–20]. Briefly, the assay quantitates fetal liver (FCFU) or bone marrow (CFU) hemopoietic colonies that have developed in the spleens of lethally irradiated (750 rad) male recipients 8 days after i.v. challenge with the stem cell inoculum. The challenge inoculum is generally 5×10^5 fetal liver or 10^5 bone marrow cells to normalize the numbers of hemopoietic stem cells in the experimental and control groups. The irradiated recipients are either previously immunized with fetal or tumor cells or transfused with sensitized LNC or immune serum.

Lymph node cell transfer

In these experiments non-immunized mice were used for the colony-forming assay. Prior to inoculation, the fetal liver (FLC) or bone marrow (BM) cells were incubated with normal or sensitized LNC to allow cytotoxic killing of susceptible stem cells. Sensitized LNC were obtained from FLC- or PCT-immune donors. A 10:1 ratio of effector to target cells was used in a 60 min incubation in heparinized vials gently agitated on a platform rocker in a 37°C (5% CO_2) incubator. The LNC's have a negligible colony-forming capacity (as opposed to spleen cells, which cannot be used as effector cells in this assay). Thus the colonies on the spleens of the irradiated recipients reflect the progeny of the remaining viable challenge FLC or BM.

A 20 min preincubation of the LNC's with either adult vs fetal liver antigen or normal vs cancer patient serum was conducted to test for blocking of cell-mediated antifetal immunity; 6×10^7 LNC were incubated with 500 μg of antigen or 0.5 ml of serum prior to their admixture with the target colony-forming cells. No corrections for viability were made in any of the samples after incubation; the colony-forming inoculum was calculated on the basis of an initial equivalent number of viable cells in each group.

RESULTS

Cell-mediated reduction of fetal colony-forming units (FCFU) by lymph node cells (LNC) sensitized to fetal liver or to a BALB/c plasma cell tumor has previously been reported by Salinas *et al.* [18]. We attempted to block this effect by preincubating the effector LNC with fetal antigen before they were mixed with target stem cells and adoptively transferred to non-

Table 1. Effect of transfer of sensitized lymph node cells (LNC) on adult bone marrow (CFU) and fetal liver (FCFU) colony formation, and blocking by fetal antigen

Experiment number	Incubation stem cells	Mean spleen-colony count ± S.E.*				
		CFU Preincubation of LNC		FCFU Preincubation of LNC†		
1		Adult liver 500 μg (KCl)	Fetal liver 500 μg (KCl)	Adult liver 500 μg (KCl)		Fetal liver 500 μg (KCl)
	No LNC	23.7 ± 2.1	22.3 ± 2.2	6.9 ± 1.2		9.2 ± 1.4
	Normal LNC	24.9 ± 1.3	23.8 ± 1.4	12.0 ± 1.5		15.1 ± 1.5
	Anti-fetal LNC	24.3 ± 2.6	22.7 ± 1.7	7.2 ± 1.1		13.1 ± 1.7
				($P < 0.025$)	($P < 0.01$)	
2		Normal BALB/c serum	FLC-immunized BALB/c serum	Normal BALB/c serum		FLC-immunized BALB/c serum
	No LNC	18.8 ± 1.8	15.8 ± 1.0	14.5 ± 1.2		16.7 ± 1.4
	Normal LNC	19.9 ± 1.2	18.9 ± 2.0	19.1 ± 1.3		19.0 ± 0.7
	Anti-Fetal LNC	18.6 ± 0.8	15.7 ± 1.0	13.9 ± 1.0		19.6 ± 1.3
				($P < 0.01$)	($P < 0.005$)	
3		Human normal serum (2.5 ng/ml)	Patient serum (CEA) (28.5 ng/ml)	Human normal serum (2.5 ng/ml)		Patient serum (CEA) (28.5 ng/ml)
	No LNC	23.4 ± 1.6	23.1 ± 0.7	16.2 ± 2.4		17.0 ± 2.2
	Normal LNC	27.3 ± 0.9	27.3 ± 1.6	16.1 ± 2.2		18.4 ± 2.4
	Anti-fetal LNC	26.2 ± 1.5	25.0 ± 2.2	9.9 ± 1.1		14.3 ± 2.1
				($P < 0.025$)	($P < 0.07$)	

*Only significant P values are shown. Challenge with LNC alone induced negligible colony formation, the mean number of colonies, regardless of preincubation, being only 0.7 ± 0.3 colonies per spleen. Ten mice per challenge group.

†Controls with no preincubation of LNC had the following values: No LNC, 16.3 ± 0.9; Normal LNC, 18.3 ± 1.9; Anti-fetal LNC, 8.8 ± 2.0 ($P < 0.005$).

immunized recipients. The data from these experiments is presented in Table 1.

There was no effect on bone marrow CFU in any of these experiments. Lymph node cells alone were shown to have negligible colony-forming capacity (Table 1, footnote *). However, both FCFU and CFU were often higher after incubation with normal LNC than in groups receiving no transfer of LNC. Enhancement of bone marrow colony formation by LNC has previously been noted, both *in vivo* [31] and *in vitro* [32]. Therefore, the statistical analysis was made between the groups receiving normal LNC (Hank's immunized) vs anti-FLC LNC (vertical comparisons in Table 1). Horizontal comparisons are included for the "No LNC" groups, showing that the fetal antigen or patient serum was not acting (at the target cell level) on the colony-forming FLC.

In the first experiment, preincubation of the anti-FLC LNC with solubilized fetal liver antigen abolished the reduction in FCFU (from 12.0 to 7.2 colonies) achieved in the presence of antigen solubilized from adult mouse liver.

Similarly, in the second experiment, preincubation of the sensitized LNC with BALB/c serum collected 24 hr after 2 daily fetal cell immunizations abrogated the reduction in FCFU (from 19.1 to 13.9 colonies) observed in the treatment with normal syngeneic serum. Controls with no pretreatment of LNC (Table 1, footnote †) showed that LNC enhancement of FCFU was independent of antigen effects, and that the preincubation of LNC with normal antigen or serum used in these experiments did not compromise the 50% reduction of FCFU achieved by sensitized LNC alone.

Since we had previously demonstrated the cross-reactive fetal antigen in human colonic carcinoma [21], serum from such a patient containing a known circulating fetal antigen was tested in experiment 3 for blocking activity against FLC-sensitized lymphocytes. The serum from a colonic cancer patient with a CEA level of 114 ng/ml (diluted 1:4 by *in vivo* absorption) was compared to normal human serum (also absorbed). The cancer patient's serum suppressed the cytotoxic action of the sensitized lymphocytes. Calculating the percent reduction in FCFU from the normal LNC control values (39% in normal serum vs 22% in patient serum), this represents a 44% blocking effect. While the reduction of FCFU by the sensitized LNC was statistically significant, the blocking effect of this patient's serum was marginal ($P<0.07$).

Therefore, a dose–response study was conducted testing sera from colonic cancer patients with varying levels of plasma CEA. Table 2 summarizes the data collated from these experiments. An inverse correlation was found between serum CEA concentration and the percent of reduction in FCFU in each experiment. Since the reduction in FCFU achieved by LNC preincubated with normal human serum in these experiments was 48–50% (data in footnote ¶), we have calculated blocking on a scale of 0–100% relative to these controls. Thus, in our assay serum containing less than 20 ng/ml of CEA was not blocking. There was a steep linear increase in blocking between 20 and

Table 2. Blocking of cell-mediated anti-fetal immunity by absorbed, heat-inactivated colonic cancer patient sera

Cancer Patient's serum‡	CEA concentration ng/ml	CEA concentration ng/10^7 LNC	Mean spleen-colony count ± S.E.† Normal LNC	Mean spleen-colony count ± S.E.† Anti-fetal LNC§	% Reduction FCFU‖	% Blocking¶
A	15.8	1.3	9.0 ± 1.1	4.3 ± 0.6	52***	0
B	21.0	1.8	10.4 ± 1.4	5.1 ± 0.8	51**	0
C	28.5	2.4	18.4 ± 2.4	14.3 ± 2.1	22	44
D	39.5	3.3	5.6 ± 0.8	5.0 ± 1.0	11	77*
E	52.5	4.4	5.6 ± 0.7	5.1 ± 0.8	9	81*
F	158.0	13.2	9.0 ± 1.1	8.2 ± 0.6	9	82**
G	210.0	17.5	10.4 ± 1.4	10.1 ± 0.9	3	94**

†Ten mice per challenge group.

‡Sera A and B are 1:10 dilutions of F and G (absorbed *in vitro*). Sera C, D and E were absorbed *in vivo*.

§Lymph node cells (LNC) were sensitized to BALB/c 15-day fetal liver cells, except for experiments D and E, in which LNC were sensitized to a syngeneic plasmacytoma known to express fetal antigen.

‖The level of statistical significance as determined by Student's *t*-test is indicated as *, $P<0.05$; **, $P<0.005$; *** $P<0.001$.

¶The % blocking was calculated relative to reductions in FCFU by the LNC treated with normal human serum. The spleen colony counts with normal or anti-fetal LNC in these controls were (A + B) 10.2 ± 1.0, 6.4 ± 1.1*; (C) 16.1 ± 2.2, 9.9 ± 1.1*; (D + E) 5.8 ± 1.0, 3.1 ± 0.8*; and (F + G) 10.2 ± 1.0, 5.7 ± 0.8***.

40 ng/ml, which thereafter appeared to level off. Two patients' sera (F and G) were studied at a tenfold dilution (A and B) showing that blocking could be reduced concurrent with dilution of CEA. Dilution of the pooled normal serum (controls, footnote ¶) did not alter the percent reduction of FCFU by sensitized LNC.

To check that the blocking of cell-mediated anti-fetal immunity by patients' sera was not a toxic effect, LNC incubated with each of the human sera were tested for their ability to carry on an unrelated biological function: the graft-vs-host reaction. In either the Boranic spleen colony assay [33] or the Simonsen assay [34], none of the sera impaired the ability of either BALB/c normal or sensitized LNC to mount a GVHR in C57 mice or their BALB/c F1 hybrids. Furthermore, none of the sera reduced FCFU at the target cell level in the absence of LNC.

Reduction of fetal colony formation by the adoptively transferred LNC was equally effective whether syngeneic fetal liver or plasma cell tumor (PCT) was used to immunize the LNC donors (see controls in Table 2). In an analogous fashion, blocking of anti-fetal immunity by colon cancer patients' sera also occurred regardless of whether the effector LNC had been sensitized to tumor or fetal tissue.

In contrast to the anti-fetal capability of LNC from mice immunized with irradiated PCT, LNC from PCT tumor-bearing mice were unable to reduce fetal colony-formation (Table 3). Tumor-bearing mice were given an inoculum of PCT that causes death of the host in 15 days. LNC were obtained from these animals at either day 7 (experiments 1 and 2) or day 14 (experiment 3). In all of these experiments the tumor-bearer LNC failed to reduce FCFU while the anti-PCT^{irr} LNC consistently gave 50% reductions in fetal colonies compared to controls using normal LNC. Experiment 1 shows that after 6 washes the anti-PCT^{TB} LNC caused a 77% reduction in FCFU. This is attributed to the removal of blocking factor, since similar washing of normal LNC did not affect fetal colony formation.

An interesting observation in these experiments was that normal bone marrow colony formation was abrogated or significantly reduced when the stem cells were incubated with anti-PCT TB LNC. Since the anti-PCT^{irr} LNC did not reduce CFU, it is unlikely that this phenomenon was due to LNC sensitization to a PCT-BM cross-reactive antigen.

These experiments were conducted without any *in vitro* incubations of the LNC with blocking factors, suggesting that the anti-fetal capacity of the LNC had effectively been blocked *in vivo* in the tumor-bearing donor.

Indeed, when tumor-bearing mice were used as the recipients for the colony-forming assay (Table 4), there was no reduction in FCFU. PCT-immunized recipients again showed a 50% reduction in fetal colony formation relative to the normal controls. It must be emphasized that in this experimental setting we are detecting an antibody-mediated reduction of colonies, since the cellular immune mechanisms have been paralyzed by the lethal whole-body irradiation used to halt endogenous hematopoiesis prior to

Table 3. Comparison of anti-fetal immunity mediated by lymph node cells (LNC) from tumor-immunized versus tumor-bearing mice

Expt. No.	Incubation of stem cells‡	Mean spleen-colony count ± S.E.† CFU	FCFU
1	Normal LNC	9.3 ± 1.0	9.0 ± 1.1
	Anti-PCT^{irr} LNC	7.1 ± 1.0	4.8 ± 0.7*
	Anti-PCT^{TB-7} LNC	0.4 ± 0.4***	8.6 ± 0.9
	Normal LNC, washed		9.2 ± 1.0
	Anti-PCT^{TB-7} LNC, washed		2.1 ± 1.7**
2	Normal LNC	14.1 ± 1.5	5.8 ± 1.0
	Anti-PCT^{irr} LNC	13.9 ± 2.1	3.1 ± 0.5*
	Anti-PCT^{TB-7} LNC	5.1 ± 0.5***	5.7 ± 1.0
3	Normal LNC	14.5 ± 1.2	12.0 ± 1.5
	Anti-PCT^{irr} LNC	14.0 ± 1.3	5.7 ± 0.8**
	Anti-PCT^{TB-14} LNC	9.7 ± 0.9*	11.5 ± 1.7

†The level of statistical significance as determined by Student's *t*-test is indicated as *, $P<0.05$; **, $P<0.005$; ***, $P<0.001$. Ten mice per challenge group. CFU and FCFU are colony-forming units from bone marrow and fetal liver, respectively, in non-immunized recipients.

‡PCT^{irr}, irradiated plasmacytoma cells used to immunize LNC donors; PCT^{TB}, sensitized LNC taken from tumor-bearing mice at day 7 or day 14 (terminal stage of disease).

Table 4. Blocking of antibody-mediated anti-fetal immunity in tumor-bearing mice

Expt. No.	Status of recipient‡	Mean spleen-colony count ± S.E.† CFU	FCFU
1	Hank's immunized	35.0 ± 0.8	13.6 ± 1.9
	PCT^{irr} immunized	34.8 ± 1.0	7.2 ± 1.2*
	PCT tumor-bearing	36.8 ± 1.6	13.0 ± 1.2
	FLC^{irr} immunized	34.6 ± 1.8	8.1 ± 1.0*
2	Untreated: challenged with normal BM	11.1 ± 1.8	
	Untreated: challenged with BM from PCT tumor-bearer	17.3 ± 1.5*	

†The level of statistical significance as determined by Student's *t*-test is indicated as *, $P < 0.05$. Ten mice per group. CFU and FCFU are colony-forming units from bone marrow and fetal liver, respectively.

‡PCT, plasma cell tumor; BM, bone marrow; FLC, fetal liver cells; stem cell recipients were either non-immunized, immunized with irradiated PCT or FLC, or tumor-bearing mice at day 7 after PCT inoculation.

challenge with the hemopoietic colony-forming cells.

In these experiments there was no antibody-mediated reduction in CFU in either the PCT-immunized or tumor-bearing animals. Furthermore, BM from tumor-bearers showed enhanced colony-forming capacity when transferred to normal recipients. These findings suggest that the CFU suppression in Table 3 may have been due to a radiosensitive suppressor cell, and that the existence of such a phenomenon is unrelated to anti-fetal immunity.

The failure of antibody-mediated anti-fetal immunity in the tumor-bearing mice in Table 4 is in contrast to the significant reduction of FCFU in both PCT- and FLC-immunized animals. The absence of blocking in the latter groups (7 days after immunization), as opposed to the serum blocking seen in Table 1 24 hr after fetal immunization, suggests the clearance of antigen following lysis of the immunizing cells. This process takes about 3 days after a s.c. tumor inoculation [35] and is probably faster by the peritoneal route used here. Since solubilized fetal antigen and cancer patients' sera were shown to be blocking in Tables 1 and 2, the mechanism most likely to account for the failure of both antibody-mediated and cell-mediated anti-fetal immunity in the tumor-bearing host is the persistance and release of fetal antigen by the growing tumor.

DISCUSSION

We have investigated the effect of various tumor-related factors upon the *in vivo* effectiveness of anti-fetal immunity. These experiments demonstrate blocking of cell-mediated reduction of fetal colony formation by (1) solubilized fetal antigen, (2) serum from recently immunized mice, and (3) serum from colonic cancer patients with metastatic disease. Furthermore, unwashed lymph node cells from plasmacytoma (PCT) tumor-bearing mice were shown to be ineffective mediators of cellular anti-fetal immunity, and antibody-mediated immunity was also shown to be blocked in these animals. These data are consistent with an antigen-shedding mechanism of immune-escape as proposed by Alexander [36].

Blocking of cell-mediated cytotoxicity at the effector level has been shown *in vitro* by tumor-associated [36–38] and embryonic [27, 39] antigens in animal tumor models. Poupon *et al.* [40] detected free circulating tumor antigen in the sera of plasmacytoma-bearing BALB/c mice. These sera could independently provoke a migration inhibition of immune spleen cells or block the reaction initiated by extraneous antigen. While sera from tumor-immunized mice were also blocking in their system, in the spleen-colony assay, adoptive transfer of LNC from PCT-immunized mice effectively reduced FCFU, whereas LNC from tumor bearers did not. This discrepancy could be due to route and timing of immunization, since we did observe blocking by serum of FLC-immunized mice 24 hr after immunization.

These observations become important in the current controversy of whether tumor-associated fetal antigens are involved in anti-tumor immunity. The evidence is conflicting in different animal models. Immunization with fetal cells has been reported to offer the host some protection against SV40, adenovirus, Rauscher virus, and methylcholanthrene (MCA) induced tumors [1–5]. However, transplantation resistance could not be induced in this way against

a mouse polyoma tumor or rat MCA sarcoma [7–13] except in the case of an MCA lung metastasis model [6]. In some MCA tumor systems [11, 13] fetal immunization has actually enhanced tumor growth. In the latter study, tumor enhancement also resulted when immunity was induced by embryo or tumor tissues grown in implanted diffusion chambers, suggesting a role of soluble embryonic and tumor antigens.

Various requirements have been delineated for successful anti-tumor fetal vaccination, including sex of recipients, route and timing of immunizations, irradiation, and choice of immunogen relative to parity and gestational phasing of the expression of onco-fetal gene products [22]. Given these considerations, we would suggest that it is important to determine that one has established effective *in vivo* anti-fetal immunity when evaluating the anti-tumor efficacy of a fetal vaccination regimen.

The FCFU assay has demonstrated cross reactivity between the BALB/c fetal liver hemopoietic stem cell and various animal tumors including BALB/c PCT, MSV-BALB/3T3, C57 BL/6 EL4, hamster SV40 [18], and BALB/c P 1798 [19], as well as several human tumors [20, 21]. A fetal immunization regimen that successfully reduced fetal colony formation in the FCFU assay has previously been shown to induce protection against the same BALB/c PCT tumor [2].

Reductions of FCFU by LNC sensitized to either syngeneic FLC or PCT were equally inhibited by human colonic cancer patients' sera with elevated CEA levels. The degree of blocking was correlated with CEA levels in the sera, and increased from 0 to 80% in the range of 20–40 ng CEA/ml of serum. It has not been conclusively established in these experiments that CEA or fetal antigen alone is responsible for the observed blocking, since immune complexes or unrelated active substances could be present in the sera. Data on the partial purification and characterization of the fetal liver–human tumor cross-reactive antigen have been reported elsewhere [20]. The relevant point regarding CEA is that in clinical screening studies [41], except for primary colonic or pancreatic carcinoma, Roche-CEA titers above 20 ng/ml were associated with metastatic disease, since blocking in the FCFU assay rapidly progressed above this level. In contrast, Baldwin *et al.* [42] found no correlation with CEA levels in the blocking of patients' lymphocyte cytotoxicity for human colonic carcinoma by papain-solubilized tumor antigen. This is not unexpected, since their assay system incorporated aspects of both anti-fetal and tumor-specific immunity.

Fetal colony reduction by LNC from FLC- or PCT-immunized mice could be abrogated by *in vitro* incubation of the effector cells with fetal antigen extract or with serum containing fetal antigens. Furthermore, neither antibody nor LNC from PCT tumor-bearers successfully reduced FCFU, but after 6 washes, PCT^{TB} LNC achieved the most potent anti-fetal activity seen in these experiments. These results suggested that both humoral and cell-mediated anti-fetal immunity may be blocked *in vivo* by circulating tumor-associated fetal antigen. If this anti-fetal immunity is important in host defense against cancer, then a mechanism by which the tumor would suppress the immune response might be the release of fetal antigen.

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Effect of Methylglyoxal-Bis (Guanylhydrazone), an Inhibitor of Spermidine and Spermine Synthesis, on Cell Cycle Traverse*

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Abstract—*Flow cytometric analysis of the cellular DNA content and high pressure liquid chromatographic analysis of the cellular concentration of the polyamines putrescine, spermidine and spermine have been performed on exponentially growing populations of rat brain tumor cells following treatment with methylglyoxal-bis(guanylhydrazone) (MeGAG). This drug has previously been found to be a potent inhibitor of S-adenosyl-L-methionine decarboxylase, the enzyme that catalyzes the initial step in spermidine and spermine biosynthesis.*

MeGAG was found to inhibit cell proliferation within one generation time. During this time it produced an increase in the fraction of cells with a 2C DNA content (cells in the G_1 phase of the cell cycle). Continued accumulation of cells in the G_1 phase occurred with sustained treatment. Drug treatment also resulted in a marked decrease in the cellular spermidine and spermine concentrations. Cells did not emerge from their G_1-arrest after the addition of spermidine or fresh medium and apparently had become irreversibly blocked by the drug.

We suggest that (1) growth inhibition by MeGAG was due to depletion of the cellular pools of spermidine and spermine, and that (2) the cells accumulated in the G_1 phase of the cell cycle because of a possible requirement for spermidine and spermine during the subsequent phases (S, G_2 and M), the period during which polyamine biosynthesis and accumulation take place in continuously dividing mammalian cells.

INTRODUCTION

METHYLGLYOXAL-BIS(GUANYLHYDRAZONE, 1,1′-[(methylethanediylidene)dinitrilo]-diguanidine (MeGAG), is a synthetic compound that possesses a significant antiproliferative capacity. In a pharmacological study of this substance, Mihich [1] observed that its inhibitory effect on cell proliferation could be prevented by concurrent administration of spermidine. This fact suggested that the effect of MeGAG might be due to interference with the metabolism of the polyamines, putrescine, spermidine and spermine. The subsequent finding by Williams-Ashman and Schenone [2], that MeGAG was a very potent inhibitor of mammalian putrescine-activated S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50), the rate-limiting enzyme in the synthesis of spermidine (and spermine) [3, 4] further substantiated this idea and opened a new avenue for obtaining information concerning the physiological role(s) of the polyamines.

The inhibition of putrescine-activated S-adenosyl-L-methionine decarboxylase activity in

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A preliminary account of these findings was presented at the 1976 Summer Meeting of the Swedish Biochemical Society (Lund, Sweden, June 10–11, 1976).

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extracts of rat ventral prostate and yeast by MeGAG [2] has since been confirmed with enzyme preparations from many other tissues [5–10]. Some information has also been obtained regarding the effect of MeGAG *in vivo*. Thus, when studying the concentrations of the polyamines in spleens of leukemic mice as affected by treatment with various antineoplastic agents, it was observed that all the drugs tested, except MeGAG, caused decreases in the concentrations of putrescine, spermidine and spermine [11]. MeGAG-treatment resulted in decreased spermidine and spermine concentrations but in an increased putrescine concentration [9, 11]. The accumulation of putrescine was due to increased L-ornithine decarboxylase (EC 4.1.1.17) activity, i.e., increased putrescine synthesis, as well as decreased S-adenosyl-L-methionine decarboxylase activity, i.e. decreased utilization of putrescine for spermidine (and spermine) synthesis [9, 12]. Similar results were obtained from other laboratories for different experimental systems [6, 8, 13].

There is considerable evidence to suggest that the polyamines are involved in the regulation of cellular growth. The biosynthesis and the intracellular levels of the polyamines increase markedly following treatment with various growth stimuli [14–16] and then decline as the cells approach a stationary phase of growth [11, 17–19]. In fact, during the growth of a rat brain tumor cell line, polyamine biosynthesis as well as the spermidine content showed high positive correlations with the cellular growth rate [19]. Furthermore, it has been found that virus-transformed cells have a higher putrescine content than their normal non-infected counterparts despite the fact that both have similar growth rates [20].

The rate of polyamine synthesis in mammalian cells changes during the cell cycle [21, 22] and the cellular polyamine content increases markedly during the S, G_2 and M phases [23–25]. As a means of studying whether spermidine and spermine are essential for progression through these cell cycle phases we have analyzed the effect of MeGAG-treatment, i.e. spermidine and spermine synthesis inhibition, on cell cycle traverse of exponentially growing populations of rat brain tumor cells *in vitro*. The distribution of the cells among the G_1, S and G_2M phases of the cell cycle, determined by means of flow cytometry at the time when population growth had ceased due to drug-treatment, showed that the majority of the cells had accumulated in the G_1 phase of the cell cycle.

MATERIAL AND METHODS

Cell culture

Rat brain tumor cells were obtained from W. H. Sweet, P. T. Kornblith, J. R. Messer and B. O. Whitman of the Massachusetts General Hospital, Boston, Massachusetts. The brain tumor was induced in CD Fisher rats by weekly i.v. injections of N-methylnitrosourea. Cell culture and preservation methods have been described by Benda *et al.* [26]. Upon receipt, the frozen brain tumor cells were thawed and suspended in Eagle's basal medium (BME) supplemented with L-glutamine (398 mg/l), fetal calf serum (10%), and antibiotics (penicillin, 80.5 I.U./ml; streptomycin, 80.5 I.U./ml), and grown in monolayer culture according to the method for continuous cell culture described by Barker, Hoshino and Wilson [27].

Growth experiments were performed in 75 cm^2 Falcon flasks as previously described by Heby *et al.* [19]. Each flask initially contained 1.0×10^6 cells in 15 ml of BME with Earle's balanced salt solution supplemented with L-glutamine (292 mg/l), fetal calf serum (10%), BME vitamin mixture (1%), BME essential amino acid mixture (1%), and penicillin–streptomycin mixture (1%). The cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

MeGAG, dissolved in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) immediately prior to the experiment, was added to cultures in exponential growth. At various times after addition of the drug, cells were harvested by trypsinization (0.25% trypsin and 0.02% Na_2EDTA in Ca^{2+}- and Mg^{2+}-free HBSS for 5 min at 37°C) and suspended in phosphate-buffered saline (PBS). To determine the number of cells per Falcon flask, an aliquot of the dispersed cell suspension was counted in a hemocytometer. Samples were then taken for flow cytometric analysis as well as for polyamine analysis.

Flow cytometric analysis

To determine the distribution of cells in the cell cycle, samples containing $1–4 \times 10^6$ cells were washed with ice-cold PBS and fixed in PBS containing 10% formalin overnight. The cells were then Feulgen-stained with the fluorescent dye acriflavine which is specific to DNA according to the method described by Gill and Jotz [28] and analyzed on the Livermore bicolor flow cytometer [29–31]. In this device the stained cells are hydrodynamically focused so that they flow at uniform velocities in single file, at rates of up to 1000 cells/sec, through an intense beam of

exciting light from an argon ion laser. The acriflavine in each cell is stimulated to fluoresce; the intensity of fluorescence is proportional to the amount of dye in the cell and is a measure of the cellular DNA content. The fluorescent light pulse from each cell is detected by a photomultiplier and converted into an electrical pulse, whose amplitude is digitized and stored in the memory of a multichannel analyzer, so that after analysis of a large number (10^6) of cells, the contents of the analyzer represents the DNA distribution of the population.

The distribution of cells among the G_1, S and G_2M phases of the cell cycle were calculated from the DNA distributions utilizing a computer-based mathematical technique [32]. The duration of each phase of the cycle could then be measured from the doubling time of the exponentially growing culture whose growth fraction was unity, assuming that the doubling time equaled the generation time and that the population age distribution decreased exponentially with age [33].

Polyamine analysis

To determine the concentration of the polyamines, the cells were sedimented by centrifugation at 500 × ***g*** for 5 min at 4°C and the cell pellet was sonicated in 10% trichloroacetic acid (approximately 200 μl per 10^6 cells). The homogenate was kept on ice for 1 hr, centrifuged at 8000 × ***g*** for 5 min, and 50 μl of the supernatant was analyzed for its polyamine content utilizing a Durrum D-500 amino acid analyzer as previously described [19]. Protein was determined according to the method described by Lowry *et al.* [34].

Chemicals

Methylglyoxal-bis(guanylhydrazone) dihydrochloride monohydrate (MeGAG) was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, and cell culture media and salt solutions were purchased from Microbiological Associates, Bethesda, Maryland and from GIBCO, Santa Clara, California.

RESULTS

Inhibition of cell proliferation by MeGAG

Rat brain tumor cells in exponential, i.e. asynchronous, growth were treated with 40 or 400 μM MeGAG and the cell number was measured as a function of time (Fig. 1). The untreated control cells grew with a population doubling time of approximately 23 hr. After the addition of MeGAG the cell number continued to increase at a rate similar to that of the control

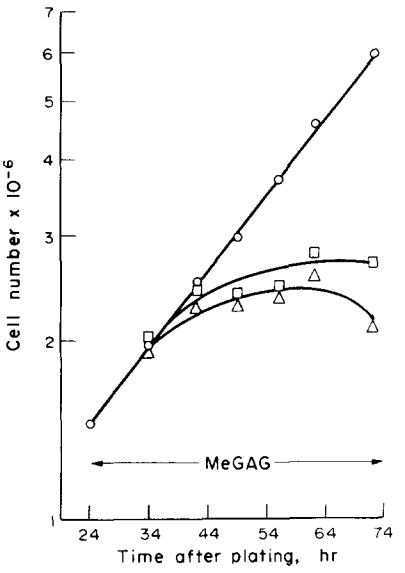

Fig. 1. Effect of methylglyoxal-bis(guanylhydrazone) on proliferation of rat brain tumor cells in vitro. *Methylglyoxal-bis(guanylhydrazone) (MeGAG) was added to exponentially growing cells, i.e.* 24 hr *after plating of* 1.0×10^6 *cells;* (○) *untreated cells;* (□) *cells treated with* 40 μM *MeGAG;* (△) *cells treated with* 400 μM *MeGAG.*

cells for about 18 hr, after which time it became almost constant. Drug concentrations lower than 40 μM reduced, but did not stop, cell proliferation after a lag of about one cell generation time (not shown). At a 400 μM concentration the drug caused a marked cell death after 48 hr.

After 24 hr of treatment most of the cells were permanently blocked from progressing through the cell cycle; cell proliferation did not resume upon resuspension in fresh growth medium or in fresh medium containing 2 mM spermidine. In the latter experiment, the cells were grown in a medium supplemented with 10% horse serum instead of fetal calf serum to avoid possible oxidation of the added spermidine. Amine oxidases which are present in bovine sera, but not in horse serum, may convert extracellular polyamines into toxic aldehyde derivatives, which may have an adverse effect on growth [35].

Accumulation of cells in the G_1 phase of the cell cycle after treatment with MeGAG

To reveal any possible cell cycle phase specificity of MeGAG, we analyzed the cellular DNA content in populations of rat brain tumor cells growing *in vitro* at various times after drug-treatment. Exponentially growing cells were treated with 40 μM MeGAG for 20, 30 and 45 hr. The cellular DNA content was determined utilizing a flow cytometric technique

[29] and the distribution of the cells among the G_1, S and G_2M phases of the cell cycle was estimated by computer analysis according to the method of Dean and Jett [32].

Figure 2 shows the fraction of cells in the G_1 phase of the cell cycle at various times after the addition of the drug. After 20 hr of treatment, the cell cycle distribution differed significantly from that of the untreated control cultures; the G_1 fraction had increased by 15%. The G_1 fraction increased continuously during the 45-hr treatment-period studied; by 30 hr it was 31% and by 45 hr 42% greater than in untreated control populations. In parallel with the increase in the fraction of cells in G_1 there was a decrease in the fraction of cells in S and G_2M. During the experimental period studied, the fraction of cells in G_1 decreased slightly in the untreated control populations.

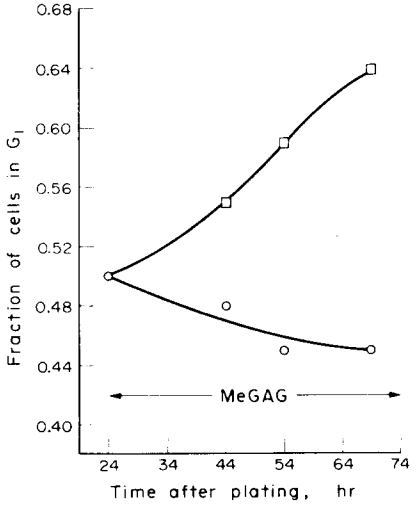

Fig. 2. Kinetics of the perturbation in cell cycle distribution following methylglyoxal-bis(guanylhydrazone)-treatment. Methylglyoxal-bis(guanylhydrazone) (MeGAG) was added to exponentially growing cells and flow cytometric analysis of the cellular DNA content was performed at various times after the addition. The distribution of cells among the G_1, S and G_2M phases of the cell cycle was calculated from the DNA distributions utilizing a computer-based mathematical technique [32]; (○) untreated cells; (□) cells treated with 40 μM MeGAG.

To study whether the cells blocked in G_1 would be able to enter the S phase upon removal of the drug, fresh medium (with and without 2 mM spermidine) was added to brain tumor cells treated for 20 hr with 40 μM MeGAG and the composition of the cell population was followed every second hour by flow cytometric analysis. No progression of cells into the S and G_2M phases was observed and it may be concluded that when cell proliferation has ceased due to MeGAG-treatment a permanent block, mainly in the G_1 phase, is imposed on the cells.

Assuming that the rat brain tumor cells, whose growth fraction was unity, had a population doubling time (23 hr) that was identical with the cell cycle time, the phase durations of untreated control cells were approximately 9.5, 9.4 and 4.1 hr, respectively, for G_1, S and G_2M (see experimental procedures). These phase durations are similar to those obtained by analysis of fraction of labeled mitoses curves derived from cells grown under the same conditions.

Accumulation of putrescine and depletion of spermidine and spermine in cells treated with MeGAG

Immediately following the addition of MeGAG, the putrescine concentration increased; by 10 hr it was maximal and twice that of untreated control cells (Fig. 3). Shortly thereafter the putrescine concentration decreased and approximately 25 hr after the addition of the drug it was below the control level.

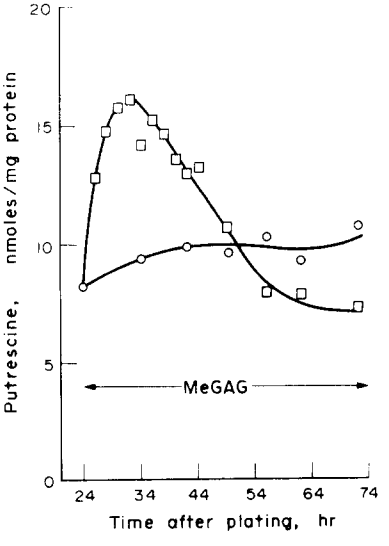

Fig. 3. Effect of methylglyoxal-bis(guanylhydrazone) on putrescine concentration in rat brain tumor cells. Methylglyoxal-bis(guanylhydrazone) (MeGAG) was added to exponentially growing cells and high-pressure liquid chromatographic analysis of the cellular polyamine concentration was performed at various times after the addition: (○) untreated cells; (□) cells treated with 40 μM MeGAG.

In contrast to the increase in putrescine concentration, there was a decrease in the cellular spermidine and spermine concentrations (Fig. 4). The spermidine and spermine concentrations started to decrease immediately following the addition of MeGAG, which shows that the drug rapidly passed the cell membrane. About 18 hr after the addition of the

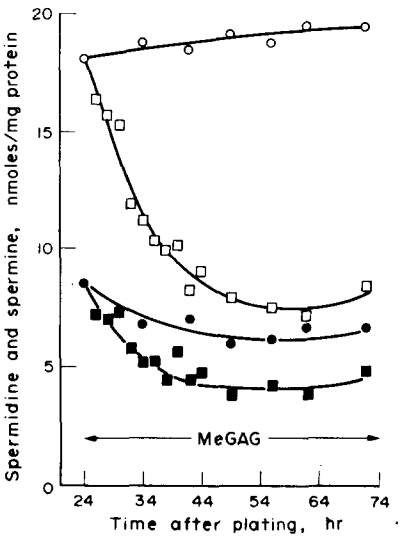

Fig. 4. Effect of methylglyoxal-bis(guanylhydrazone) on spermidine and spermine concentration in rat brain tumor cells. Methylglyoxal-bis(guanylhydrazone) (MeGAG) was added to exponentially growing cells and high-pressure liquid chromatographic analysis of the cellular polyamine concentrations was performed at various times after the addition: (○, ●) untreated cells; (□, ■) cells treated with 40 μM MeGAG. (Top) Spermidine; (Bottom) Spermine.

drug the cellular spermidine concentration had decreased by 50% and that of spermine by 35% relative to their controls. The lowest concentrations obtained were only 35 and 57% of the control levels for spermidine and spermine, respectively.

DISCUSSION

MeGAG inhibits growth of many experimental tumors, e.g. L1210 lymphoid leukemia [1, 11, 36–38] and sarcoma 180 ascites tumor [37]. It also has clinical activity against acute myelocytic leukemia [39–44] and various other cancer forms [39, 41, 42, 45–47]. However, these effects have only been established through survival studies on tumor-bearing mice and on patients treated with MeGAG. Thus far there have been no reports in the literature concerning the time required before population growth is arrested by treatment with this drug. To determine the duration of this possible lag period, we studied the proliferation of exponentially growing rat brain tumor cells following treatment with MeGAG.

The rat brain tumor cells used, which have a cycle time of 23 hr, ceased to divide about 18 hr after the addition of the drug, i.e. before the entire population had doubled (Fig. 1). In view of the fact that MeGAG affects the metabolism of the polyamines within 2 hr (Figs. 3 and 4) we consider it unlikely that the lag time is the result of a slow rate of uptake of the drug. Furthermore, MeGAG has been found to accumulate rapidly in a large variety of cells [48, 49].

The fact that the growth curve showed no effect of MeGAG for 18 hr does not necessarily imply that the cells took this long to respond to the drug. Flow cytometric data show that there is almost an immediate effect on cell cycle traverse; cells begin to accumulate in the G_1 phase of the cell cycle. This suggests at least a partial block in the G_1 phase, and the cells which have passed this stage will complete the cycle and eventually become blocked in G_1 of the subsequent cycle. Thus, the growth curve will not reflect the block for many hours (18 hr) but the fraction of cells in G_1 will respond immediately.

These results are at variance with those obtained by Fillingame, Jorstad and Morris [50] on concanavalin A-stimulated lymphocytes. Their data suggest that cells progress normally from G_0 through G_1 and into S, but that there is a prolongation of the S phase in the presence of MeGAG. Possibly, the fact that only a partial block (60% inhibition of DNA synthesis and cell division) was obtained and that the total polyamine content was unchanged in the lymphocyte cultures may explain their different kinetic behavior. However, no satisfactory explanation for these discrepancies can be given at the present time.

At the time when the rat brain tumor cells had ceased to divide due to MeGAG-treatment there was a residual S and G_2M population (Fig. 2). The presence of these cells, not blocked in G_1, might be explained by the following possibilities: (1) they may have escaped from the G_1 block and this could be an infrequent but regular phenomenon, (2) they may represent a subclass that is drug-resistant, (3) they may be dying, and/or (4) they may be subject to a secondary block in another part of the cell cycle. None of these possibilities can be definitely excluded at the present time, however. The fact that cells which had been subject to 24 hr drug exposure were unable to cycle normally upon resuspension in fresh growth medium suggests that the majority of the cells were irreversibly blocked and that these cells might eventually die. In contrast to this, the effect of MeGAG on phytohemagglutinin-stimulated lymphocytes could be reversed by washing the cultures free of the drug. However, this reversal took place only during the first 24 hr following its addition, the time during which the lymphocytes had not yet replicated their DNA [7].

During the first 20 hr of treatment with MeGAG the spermidine and spermine concentrations decreased by 65 and 43%, re-

spectively, whereas the putrescine concentration showed a 100% increase and a subsequent decline to the control level (Figs. 3 and 4). Inasmuch as low concentrations of MeGAG appear to exert no direct inhibitory effect on events such as DNA, RNA and protein synthesis [13, 51] but rather specifically inhibit spermidine and spermine synthesis, it seems likely that the marked decrease in spermidine and spermine concentration (as a result of the drug treatment) is the direct cause of the cell cycle arrest. Thus, the intracellular concentration of these compounds may be critical for cell cycle traverse. Since the ensuing G_1 block was irreversible, the spermidine and spermine deficiency appears to have introduced errors in the reproductive apparatus. In fact, during polyamine-starvation of polyamine-deficient mutants of *E. coli* apparent aberrations in chromosome replication [52] and in phage DNA synthesis [53] have been observed.

We have previously shown that polyamine synthesis increases markedly during two discrete phases of the cell cycle; immediately before DNA replication and before division [22, 25]. This finding indicates the phase(s) of the cell cycle during which the polyamines may exert their effect(s). Our present observation that the polyamine-depleted rat brain tumor cells are primarily blocked in the G_1 phase of the cell cycle suggests that DNA replication in particular may require spermidine and spermine. The possible requirement of polyamines for DNA synthesis is also indicated by other reports. Kay and Pegg [7], Fillingame and Morris [13], Otani *et al.* [54] and Fillingame *et al.* [50] observed that when MeGAG was added to lymphocyte cultures at the same time as the transforming agent (phytohemagglutinin or concanavalin A) the stimulation of DNA synthesis some 48 hr later (incorporation of ^{3}H-thymidine into DNA) was almost completely inhibited. However, the drug had less of an inhibitory effect when added at a later time, and when added during the DNA synthetic period the drug did not have any effect on DNA replication [50, 54]. These observations could be partly due to drug-interference with the transforming agent. However, in view of the present findings it seems likely that the effects observed are due to polyamine depletion.

Even though MeGAG can effectively inhibit DNA polymerase *in vitro* at mM concentrations [51] it does not seem likely that DNA synthesis proper is affected by the concentrations used in most studies. RNA and protein synthesis also appear to proceed unabated in the presence of the drug. In concanavalin A-stimulated lymphocytes, the RNA that accumulated in the presence of MeGAG (in the absence of spermidine and spermine accumulation) was qualitatively and quantitatively indistinguishable from that obtained in the absence of the drug (in the presence of spermidine and spermine accumulation) [13]. The reduced rate of ^{3}H-uridine and of ^{14}C-phenylalanine incorporation into RNA and protein observed by Kay and Pegg [7] in transforming lymphocytes was probably due to secondary, pharmacological effects of the high doses used, since Fillingame and Morris [13], who used much lower doses of MeGAG, observed a normal accumulation of RNA and protein in the absence of spermidine and spermine accumulation.

In view of these findings, it seems likely that depletion of the cellular spermidine and spermine content was the direct cause of the inhibition of DNA replication and cell division. The present work demonstrates through kinetic analysis that cells exhibiting exponential growth (continuously dividing) at the time of addition of MeGAG lose their capacity to synthesize DNA and to divide. As previously mentioned it was not possible to reverse the effect by washing out the drug with fresh growth medium when the rat brain tumor cells had ceased to divide as a result of treatment with MeGAG, nor was it possible to reverse the effect of the drug by adding spermidine. In experiments in which spermidine or spermine was added at the same time as MeGAG [1, 7, 54] or within 24 hr, i.e. before DNA synthesis [50], drug action was found to be completely reversible, but the later the addition of spermidine the less pronounced was its effect [54].

The inhibitory effect of a single injection of MeGAG on spermidine synthesis has been shown to persist for maximally 20 hr in the liver and kidney of the rat. This period was not sufficient to produce a large decrease in the tissue spermidine concentration [6, 8]. In fact, only a minor decrease in spermidine concentration can be expected to occur within this time period even after complete inhibition of spermidine formation since spermidine is catabolized very slowly (half-life of 4–5 days in the liver). Within the same time period in a proliferating cell system, represented by the exponentially growing rat brain tumor cell culture, the spermidine and spermine concentrations decreased by 50 and 35%, respectively (Fig. 4). This is the first occasion where a marked decrease has been observed in these parameters upon MeGAG-treatment. This decrease is due mainly to the fact that a large number of cells were able to go through one division in the absence of spermidine and spermine synthesis, thus decreasing

the cellular concentrations of these polyamines accordingly. The difference in rate of decrease between spermidine and spermine may be related to the fact that spermine is catabolized more slowly than spermidine. If the half-lives of the polyamines are as long as previously indicated one would expect that after the cells had stopped dividing the spermidine and spermine concentrations would be almost constant. Indeed this was the case in the studies we have described.

In a recent publication, Mamont *et al.* [55] reported that (DL)-α-methyl ornithine, a potent competitive inhibitor of L-ornithine decarboxylase, blocks DNA synthesis and cell proliferation of rat hepatoma cells in culture. This finding lends further support to the concept that polyamines play an essential role in the traverse of the cell cycle.

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Therapeutic Controlled Trial in Ewing's Sarcoma

Report on the Results of a Trial by the Clinical Cooperative Group on Radio- and Chemotherapy of the E.O.R.T.C.

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Abstract—*Sixty-five patients, 1–25 yr old, have been included between 1965 and 1972 in a therapeutic controlled trial comparing radiotherapy alone, to radiotherapy plus a chemotherapy giving one course of phenylalanine mustard, followed by continual administration of small doses of cyclophosphamide for at least 2 yr.*

The disease-free survival, chosen as criterion, was the same in both arms. No statistical significant difference was found in the prognosis, according to age, pathological subtype or localisation.

A much more aggressive chemotherapy is now being tested in Ewing's sarcoma with promising early results.

INTRODUCTION

In spite of its good local curability, Ewing's sarcoma keeps a bad prognosis, due to early dissemination in lungs and bones [1, 2]. The trial described below was an early tentative to evaluate the effect of chemotherapy, in addition to local radiotherapy for these patients.

The trial, sponsored by the E.O.R.T.C. (radiotherapy and chemotherapy group) has been run between June 1965 and June 1972 for inclusion of patients.

Three Centers participated:

—Institut Gustave-Roussy, Villejuif/Paris (Coordinator: Dr. O. Schweisguth).

—Rotterdam Radiotherapeutisch Institut, Rotterdam/The Netherlands (Coordinator: Prof. B. Van der Werf Messing).

—Antoni Van Leeuwenhoek Ziekenhuis, Netherlands Cancer Institute, Amsterdam/The Netherlands (Coordinator: Prof. K. Breur).

MATERIAL AND METHODS

1. Criteria for inclusion in the trial were the following:

—localized Ewing's sarcoma in any site (primary in the rib with pleural effusion were excluded).

—absence of contra-indication, by clinical, geographical or haematological status, to long term chemotherapy.

—Pretreatment evaluation including:

X-rays of primary tumor, skeleton and lungs.

Bone marrow examination.

Complete blood count and sedimentation rate.

VMA and HVA urinary excretion.

—Histopathological diagnosis, reviewed by a board of pathologists*.

Included diagnoses were typical Ewing's sarcoma, atypical or uncertain Ewing's sarcoma, reticulum cell sarcoma of bone. Five "other" cases, which the board did not consider to belong to one of these groups, were still included, because they were considered as Ewing or reticulum cell sarcoma by the local pathologist.

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*Dr. R. Van der Heul and Dr. R. Blaché.

2. Local treatment was the same in both arms of the trial: radiotherapy with a cobalt Unit to the whole of the involved bone, including soft tissue tumor and, if necessary, the adjacent joint. The given dose was 6000 rads in 6 weeks and 30 fractions, to the long bones. To short and flat bones, a dose of 4000–4500 rad was delivered to the whole of the bone with a boost of 1000–2000 rad on a smaller vol including the tumor itself. Doses for all patients are shown in Fig. 1.

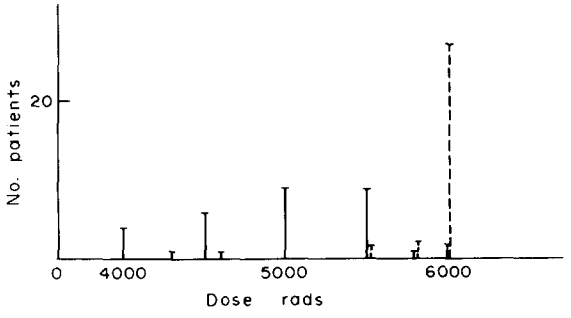

Fig. 1. Radiotherapy of the primary tumor. Doses in rad. ——— *34 flat and short bones (1 dose unknown).* – – – – *31 long bones (1 dose unknown).*

Randomization into 2 groups took place half way during the course of radiotherapy of the primary, so after 3000 rad: 1—no chemotherapy (C−) 2—adjuvant chemotherapy (C+).

Chemotherapy consisted of a course of 0.2 mg/kg/day for 10 days of phenylalanyl mustard. After recovery of haematological depression, cyclophosphamide, 2 mg/kg every other day, was given in the long run for at least 2 yr, or until relapse, without serious toxicity.

Clinical and radiological work-up were done at 2, 4, 6, 9, 12, 18 and 24 months after randomization, then every six months.

Recurrence-free survival was chosen as the comparative criterion.

3. Seventy-four patients, 1–25 yr old, were randomized during this 7 yr study (Table 1). Nine cases were excluded afterwards: in 6, metastases occurred during the course of radiotherapy (3 in each arm); in 3 there was pleural effusion complicating a rib tumor (C-arm).

Table 2 shows the repartition of patients by Center, and Table 3 by site of the primary tumor. Sex ratio, and mean age at diagnosis did not differ in both arms: the mean age in group C− was 11 yr and in group C+ 13 yr.

Nevertheless, there were more patients younger than 10 in the (C−) arm, than in the (C+) arm ($P<0.05$) (Table 4). No explanation could be detected for this difference, nor any cause for selection found.

Table 1. Distribution of the 65 patients

	Rx	Rx+C	Total
No. of randomized patients	36	38	74
Discarded after randomization	6	3	9
Definitively included	30 (46%)	35 (54%)	65

Table 2. Origin by center

	Rx	Rx+C	Total
Villejuif	24 (46%)	28 (54%)	52
Rotterdam	5	6	11
Amsterdam	1	1	2
	30	35	65

Table 3. Distribution of the patients by site of the primary tumor

	Long bones	Flat and short bones: Rib	Vertebra	Pelvic +other	
Rx	14 (45%)	3	6	7	30
		16	(47%)		
Rx+C	17 (55%)	4	3	11	35
		18	(53%)		
Total	31	7	9	18	65
			34		

Table 4. Distribution of the patients by age at diagnosis

	<10 Y.	10–19 Y.	≧20 Y.	Total
Rx	14 (64%)	15 (38%)	1	30
Rx+C	8 (36%)	24 (62%)	3	35
Total	22	39	4	65

Histopathological repartition is given in Table 5. Diagnosis in Ewing's sarcoma can be difficult, specially if biopsy material is not technically perfect which happens if patients are referred to the Centers after biopsy. In our previous experience in children, as well as in others [3, 4] natural history of the disease as well as prognosis is similar in reticulum cell sarcoma and in Ewing's tumor. In this trial, different types are evenly distributed in both arms. Two out of three of cases are reported as typical Ewing's sarcoma.

Table 5. *Distribution of the patients by histopathology*

	Typical Ewing	Atypical Ewing	Uncertain Ewing	RS	Other	Total
Rx	17 (41%)	4	2	3	3	29 (45%)
Rx+C	24 (59%)	2	1	6	2	35 (55%)
Total	41	6	3	9	5	64
						Unknown = 1

RESULTS

Seventeen out of sixty-five patients remain in first remission after 3–10 yr.*

As shown in Fig. 2, recurrences are evenly distributed in both arms of the trial and the disease free survival curve is the same. Table 6 gives the site of recurrence, known in 43/48 cases.

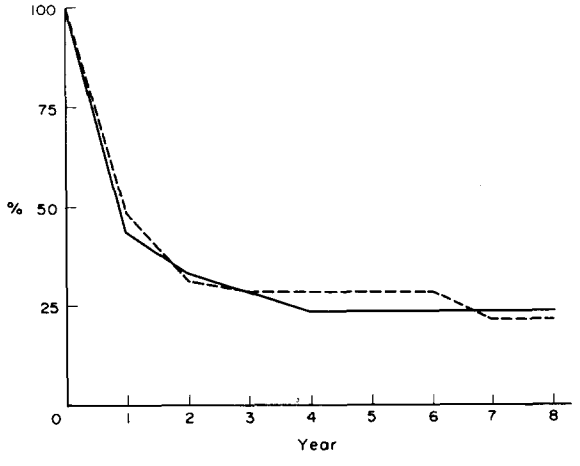

Fig. 2. *Disease-free survival.* ——— *Without chemotherapy* (30 *cases*) – – – – – *With chemotherapy* (35 *cases*).

Local relapse occurred in 6 children. Table 7 shows in these cases the site of primary tumor and the dose of radiation to the tumor. It must be noticed that only two long bone tumors relapsed despite a 6000 rad dose. Other cases were located in ribs, iliac bone and vertebra, where radiotherapy to high dose is more difficult to achieve.

Table 6. *Site of the* 48 *recurrences*

	Rx	Rx+C	Total
Local recurrence	1	5	6
Metastases: Lung	11	11	22
Bone	3	4	7
Lung and bone	5	1	6
Lymph nodes	0	1	1
CNS	0	1	1

Table 7. *Six local relapses*

Patient	Age (yr)	Primary site	Radiotherapy (rad)	Relapse time to recurrence (months)
C. Mathieu	$4\frac{1}{2}$	Vertebra C_7	4600	15
L. Marylin	1	Rib	5000	3
R. Annie	18	Radius	6000	29
S. Bruno	$4\frac{1}{2}$	Rib	5500	12
M. Pierre	$15\frac{1}{2}$	Humerus	6000	20
H. Julie	19	Pelvis	4000	4

Fifty-five percent of the metastases were detected in the 1st yr, 68% into 2 yr, the longest interval in the group being 33 months.*

Figures 3, 4 and 5, indicate the survival rates according to age, site and pathological type, neither of them showing significant differences.

As shown in Fig. 6, comparing disease-free survival and survival, death occurs rather rapidly after relapse in most of the cases. Metastatic patients have been treated with more powerful chemotherapies without a significant prolongation of life.

DISCUSSION

The clinical trial as performed by our cooperactive group, indicates that an adjuvant chemotherapy regimen with phenylalanyl mustard and cyclophosphamide in the time and dose schedule as described, failed to improve prog-

*Since the writing of this paper two other patients have relapsed:
1 local recurrence at 57 months (C–),
1 mediastinal nodes metastases at 38 months (C+),
2 have disclosed an osteogenic sarcoma in an irradiated area after 10 and 11 yr (2 C+).

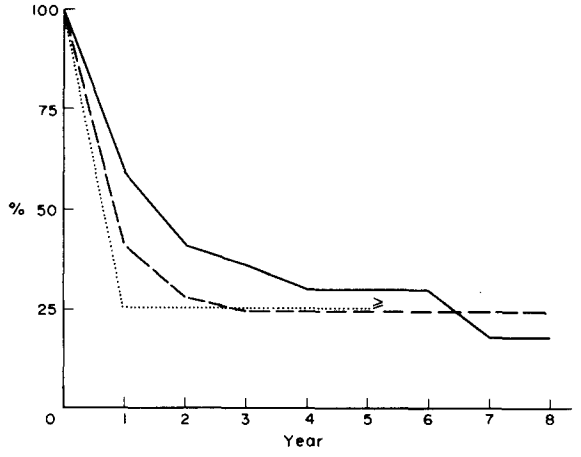

Fig. 3. Disease-free survival by age at diagnosis. ..... ≧20 yr (4 *cases*) – – – – – 10–19 *yr* (39 *cases*) ——— < 10yr (22 *cases*).

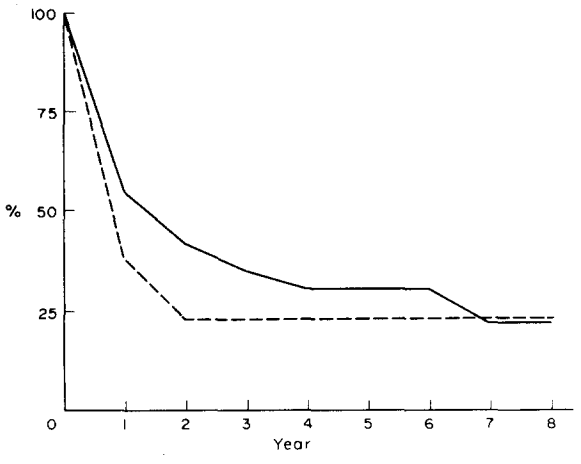

Fig. 4. Disease-free survival by site of the primary tumor. ——— *Long bones* (31 *cases*) – – – – – *Short and flat bones* (34 *cases*).

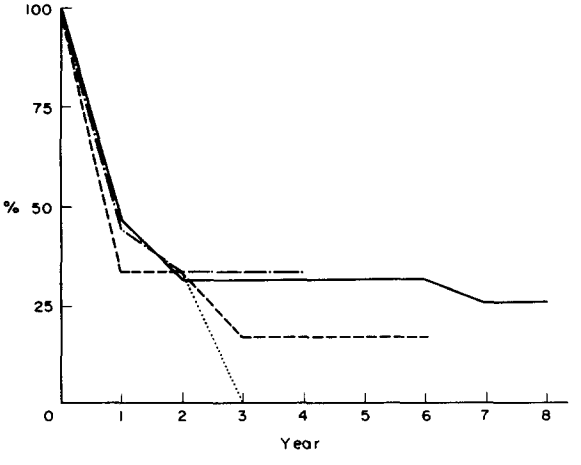

Fig. 5. Disease-free survival by histopathology. ——— *Ewing typical* (41 *cases*) – – – – – *Ewing atypical* (6 *cases*) ..... *Ewing uncertain* (3 *cases*) .–.–.–.–.– *Reticulum cell sarcoma* (9 *cases*) (5 *other and* 1 *unknown not included in the curves: see Table* 5).

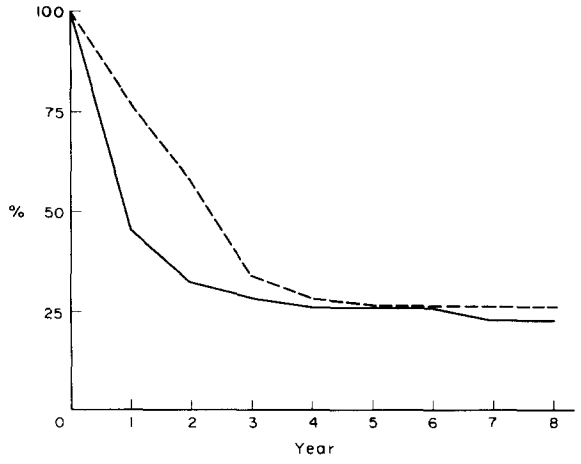

Fig. 6. Actuarial survival curve. ——— *Disease-free survival,* – – – – *Survival.*

nosis of patients suffering from clinically localized forms of Ewing and reticulum cell sarcomas.

This trial was designed 12 yr ago. With our present knowledge, it is obvious that this form of chemotherapy is inadequate, both with regard to dose and choice of the drugs.

Since 1972 it has become general practice to administer more active multiple drug chemotherapy to these patients [5–9].

The small number of cases included in 7 yr with 3 active Centers shows the difficulty, in that sort of very rare tumor, to gather a sufficient number of patients to carry through a randomized trial in a few years.

Regarding the local treatment, the type of radiotherapy given to our patients with an homogeneous protocol, shows its efficacy in 90% of the cases (6/65 local relapses). It has also given an apparent cure in 25% of the children. The price is an important growth disturbance in these patients, most of them being children in active stage of growth.

In this group of young patients, we can also confirm that there is no evolutive difference between the distinctive patterns of Ewing's Sarcoma and the so called reticulum cell sarcoma of bone.

The whole group of the patients in this trial has been treated locally in a very homogeneous and satisfactory way, and we don't expect to change local treatment [10, 11]. Since a few years, pilot studies were performed by members of our group applying for these cases cyclic multiple drug chemotherapy (Vincristine, Cyclophosphamide, Adriamycin and Procarbazine) in high dosage during 18 months, following the same local treatment with radiation. In view of

the rarity of these tumors, it seems admissible to use the data of this trial as a historic series for matched comparison. Thirty children have already been included in this current historical trial since March 1973. In June 1976, 18 of them (60%) had no evidence of disease 3–38 months after the beginning of treatment (median: 25 months). These early results seem to be promising [12] since the difference between disease free survival curves of the two series is significant up to 30 months.

Acknowledgements – We are indebted, for their most valuable contribution, to Dr. Blache and Dr. Van der Heul, pathologists of the board, and Dr. D. Sarrazin radiotherapist of the IGR.

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Relationship Between Tumour Growth Rate and Proteic Variations in Interstitial Subcutaneous Fluid and Serum: Possible Thymic Control*

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Abstract—*Some murine tumours stimulate the proliferation of target cells in diffusion chambers implanted subcutaneously near the tumour. The growth of tumours with or without the property to stimulate the proliferation of target cells was studied in relation to the proteic variations in interstitial subcutaneous fluid and serum in normal hosts and thymectomized or thymus-deprived hosts.*

"Stimulating" tumours were found to exhibit a much faster growth than "non stimulating" tumours, in normal hosts. A significant protein decrease was observed in interstitial fluid of mice bearing "stimulating" tumours as compared to control mice.

In contrast, the analysis of interstitial fluid and serum of mice bearing "non stimulating" tumours revealed a significant proteic increase.

The study of the growth behaviour of these tumours in T-cell deprived mice revealed a deceleration of the "stimulating" tumours, and a heavy acceleration for the "non stimulating" tumours. No proteic variation as compared to control mice was found in interstitial fluid and serum of tumour bearing T-cell deprived mice.

The hypothesis was formulated that some relationship exists between tumour growth rate and proteic variations in interstitial subcutaneous fluid and serum which might be under thymic control.

INTRODUCTION

In a previous work, it was found that tumour cells in diffusion chambers implanted close to some chemio-induced tumours of mean size proliferated more rapidly than in control mice. Other tumours do not show this stimulation phenomenon [1]. The analysis of interstitial fluid near these "stimulating" tumours has shown a proteic decrease, but no notable variation in the serum nor in the interstitial fluid of "non stimulating" tumours [2].

It seems of interest to correlate "stimulating" or "non-stimulating" tumours with their growth *in vivo* and with proteic variations in interstitial fluid and serum, extending the previous study to the larger tumours.

Furthermore, several investigators have reported that most of the tumours grow significantly faster in normal mice than those in thymus-deprived mice [3, 4]. This was demonstrated to be due to the lack of normal T cell function in these animals and it may be concluded that T cells are required for an optimal growth of the tumours. Existence of T-lymphocyte inhibitors has been found in the serum of tumour-bearing mice [5].

In this paper, we study the growth of two "stimulating" and two "non-stimulating" tumours in normal and T cell-deficient mice according to proteic variations in interstitial subcutaneous fluid and serum.

MATERIAL AND METHODS

Animals

C3H/He and CBA mice were obtained from breeding colonies in our laboratory, as were the nu/nu mice of Balb/c background.

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C3H/He and CBA mice were used when 2 or 3 months old, nu/nu mice when 4 or 6 weeks old.

ATXBM mice (adult, thymectomized, irradiated, bone-marrow reconstituted mice) were also used. They were thymectomized when 1 month old, lethally irradiated (850 R) 1 month later, and reconstituted by i.v. injection of 10^7 bone–marrow cells; they were used 1 month later.

Tumours

The tumours were rhabdomyosarcomas induced by intramuscular injection of methylcholanthrene. RV2 and VMM2 tumours, induced in C3H males, were respectively used from graft-passage 100 to graft-passage 115 and from graft-passage 42 to graft-passage 55. MC2 and MC3 tumours, respectively induced in CBA males and CBA females were used from graft-passage 16 to graft-passage 26.

Growth of the tumours

Comparison of the tumour growth was made after a s.c. injection of 10^5 tumour-cells, or of 1 mm^3 of calibrated tumour-fragment, varying with the experiments, in groups of 10 mice. The tumours developed in 100% of the animals. Tumour size was measured with a vernier caliper. The largest diameter and the diameter perpendicular to the largest one were determined and the average was recorded. The measurements were stopped when the first animal of each group died.

Collection of interstitial s.c. fluid and serum

The interstitial s.c. fluid was collected by means of diffusion chambers which were constituted by a plexiglass ring limited by two Millipore filters of 0.1 μ porosity. The diffusion chambers were impermeable to cellular penetration. In our experiments, they were filled with culture medium and were s.c. inserted into the back, close to the tumour of mean dia 25 mm also into the backs of the normal mice. Five days later, the chambers were removed and liquids were collected. Blood was collected from the retro-orbital sinus of normal and tumour-bearing mice. It was allowed to clot overnight at 4°C; serum was then separated and centrifuged.

Determination of the protein amounts

Total protein content of the interstitial fluid and the serum was determined according to Lowry's method [6] using crystalline bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis

Serum and interstitial fluid were subjected to electrophoresis on gradient gel slabs. The electrophoresis was performed with a Uniscil Electrophoresis Apparatus UE4 from Universal Scientific Ltd., London, U.K., loaded with four Gradipore polyacrylamide gradient gels (2.5–28%) from Gradient Pty, Ltd., Sydney, Australia. The gel slabs were prerun for 15 min at 125 V (constant voltage) to remove any excess catalyst. After the application of the samples (5 μl mixture made of 10 μl serum or interstitial fluid in 25 μl sucrose), six samples per gel slab, the gels were run first at 70 V during 20 min and then at 130 V for 15 hr. The buffer temperature was maintained at 14–15°C. The gel slabs were then stained during 30 min in 0.7% Amidoblack 10B in 7% acetic acid and destained in a Uniscil destainer apparatus UDSI. The gel slabs were scanned with a Vernon photometer integrator (42, rue des Haies, 75020 Paris, France).

Protein identification

Albumin was localized in regard to the position of the mouse albumin-purified protein. To identify the transferrin after electrophoresis, the gel slab was sliced into two parts. One part was stained and then juxtaposed the other part. The supposed transferrin fraction was sliced with a razor blade and elution from the gel section was made by electrophoresis. After concentration, the protein was identified by immunoelectrophoresis against a rabbit antimouse transferrin specific anti-serum from Cappel Laboratories.

RESULTS

Comparison of the growth-curves of "stimulating" and "non stimulating" tumours

The growth of tumours which do or do not possess a stimulating action on target cells put in diffusion chambers has been studied. Groups of 10 mice were inoculated s.c. with 10^5 syngeneic tumour-cells. Four host–tumour systems were studied: C3H male-RV2 tumour, C3H male-VMM2 tumour, CBA male-MC2 tumour and CBA female-MC3 tumour. The mean diameter of tumours of 10 mice of each group $\pm$ S.E. is given in Fig. 1 according to the number of days after the graft.

A significant difference exists between the growth-rate of RV2 and MC3 with respect to that of MC2 and VMM2. RV2 and MC3 which are "stimulating" tumours have a faster growth-rate than MC2 and VMM2 tumours which are "non stimulating" tumours.

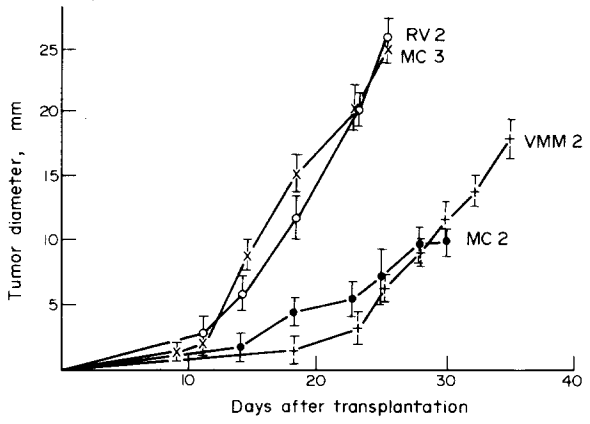

Fig. 1. Growth curve of the tumours MC2 ●, MC3 ☆, VMM2 ★, RV2 ○ in syngeneic system after a subcutaneous injection of 10^5 tumor cells. Each point represents the mean tumor diameter for a group of 10 mice ± S.E.

Variation of protein quantities in interstitial s.c. fluid and in serum of normal and tumour-bearing mice

The 4 host-tumour systems previously described have been tested. For each experiment, electrophoresis of interstitial fluid of 3 normal mice and 3 mice bearing tumours (of mean diameter 25 mm) were made on the same polyacrylamide gradient gel slab. Four experiments gave similar results. Figure 2 reproduces the curves obtained after scanning of the gel slabs of interstitial fluid of a normal CBA and of MC2- and MC3-tumour-bearing CBA's. Four main zones were noted. Zone C contained predominantly transferrin followed by α-globulins. Zone D contained albumin and pre-albumin. For the MC2 tumour, a large increase was observed in zones A and C. Similar results were found for the VMM2 tumour. By contrast, for the MC3 tumour, a decrease of proteins in the same zones was observed with similar results for the RV2 tumour.

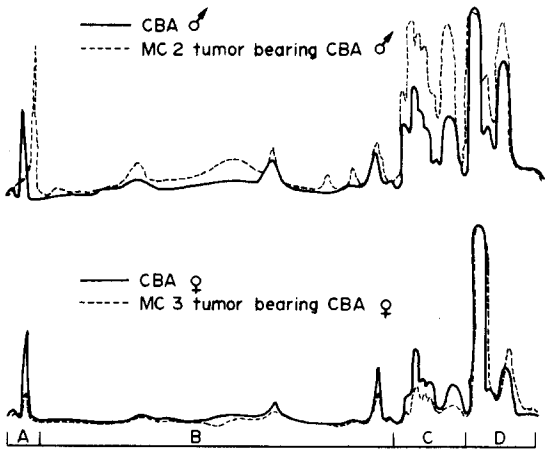

Fig. 2. Superimposed curves of polyacrylamide gradient gel electrophoresis of the interstitial fluid of normal mice and tumour-bearing mice: diameter 25 mm.

Identical work was performed with the serum. An increase of proteins was found for MC2 and VMM2 tumours and a decrease for RV2 and MC3 tumours. Figure 3 reproduces the curves obtained after scanning of the gel slab of serum of normal CBA and of MC2- and MC3-tumour-bearing CBA's.

It can be seen that the changes are more considerable in interstitial fluid than in serum. Indeed, for serum, in all experiments, no notable variation was found for zone A but only for zone C.

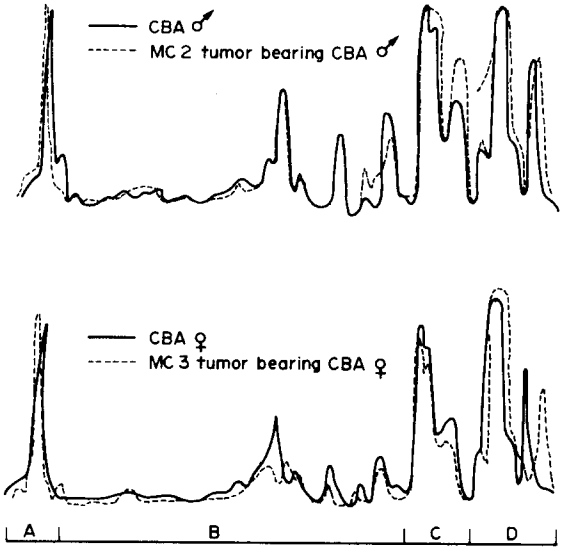

Fig. 3. Superimposed curves of polyacrylamide gradient gel electrophoresis of the serum of normal mice and tumour-bearing mice: diameter 25 mm.

As it was difficult to quantify these observed differences with the electrophoresis technique, an estimate of total proteins in the serum and interstitial liquid of all tested systems has been made according to Lowry's method [6]. Protein amounts of groups of 6 control mice and 6 tumour-bearing mice having mean tumour dia 25 mm have been calculated according to Lowry's method [6]. The mean quantity of proteins in g/100 ml ± S.E. has been recorded for each group of 6 mice and Student's *t*-test has been made to compare results obtained on control and tumour-bearing mice. Results are given in Table 1 and they show good correlation with those found with the electrophoresis method. In interstitial fluid of RV2 and MC3 tumour-bearing mice, there was a significant decrease of proteins with respect to control mice and an increase for MC2 and RV2 tumour systems. Differences were also observed for serum but they were only significant in serum of "non stimulating" tumour-bearing mice.

These results corroborate those previously

Table 1. Estimation of total proteins of interstitial fluid and serum of normal and tumour-bearing mice according to Lowry's method

Mice and tumours	Interstitial fluid		Serum	
CBA ♀	2.90±0.25*		4.50±0.30	
MC3 tumour-bearing CBA ♀	2.21±0.41	$P<0.05$†	4.82±0.41	NS‡
CBA ♂	3.09±0.03		4.71±0.05	
MC2 tumour-bearing CBA ♂	3.60±0.23	$P<0.05$	5.33±0.13	$P<0.05$
C3H ♂	3.12±0.42		5.03±0.33	
RV2 tumour-bearing C3H ♂	2.55±0.35	$P<0.05$	4.61±0.12	NS
VMM2 tumour-bearing C3H ♂	3.86±0.12	$P<0.05$	5.54±0.37	$P<0.05$

*Mean amount of proteins in g/100 ml ± S.D. contained in interstitial fluid and serum of 6 normal mice and 6 tumour-bearing mice (mean tumour diameter 25 mm).
†Statistical differences between normal mice group and tumour-bearing mice group are evaluated by Student's *t*-test.
‡NS: not significant.

published [2], i.e. a decrease of proteins in interstitial fluid of mice bearing "stimulating" tumours. Extending the investigations to large tumours, a significant increase of proteins in interstitial fluid and serum is revealed in "non stimulating" tumours.

Influence of "stimulating" and "non stimulating" tumours on the tumour growth in vivo

To demonstrate a direct relationship between tumour growth and proteic variations in interstitial fluid, we wanted to see first whether the growth of "stimulating" tumours was influenced when they were implanted in mice bearing a "non stimulating" tumour, and second, whether the growth of "non stimulating" tumours was also influenced by their implantation in animals bearing "stimulating" tumours. For this purpose, the following experiments were carried out. A ten CBA ♀ mice group was subcutaneously grafted with a 1 mm^3 fragment of MC3 tumour in the tail direction. When the mean tumour diameter reached 10 mm, a 1 mm^3 fragment of MC2 tumour was injected in the head direction in the same mouse and in the 10 control mice. Similar experiments were performed with MC3 tumour grafted in MC2 tumour-bearing mice and control mice. The growth curves of MC2 and MC3 tumours in normal and MC3 and MC2 tumour-bearing mice respectively, are recorded in Fig. 4. When mice carried the MC3 tumour, the growth of the MC2 tumour was significantly accelerated as compared to that of the control ($0.05<P<0.001$). On the contrary, when mice carried the MC2 tumour, the growth of the MC3 tumour was slowed down ($0.05<P<0.01$).

It is possible to hypothesize that the protein

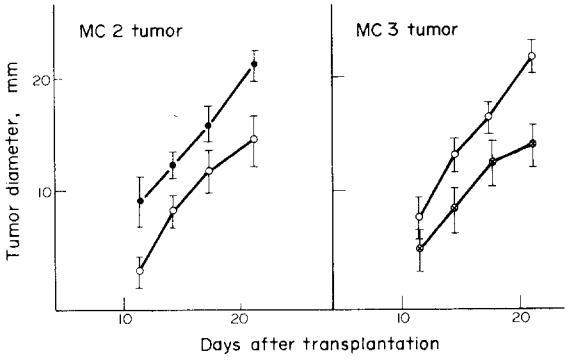

Fig. 4. Growth of MC2 and MC3 tumours grafted in normal CBA mice (○), and respectively in MC3 (●), and MC2 (⊗) tumour-bearing mice.

decrease in interstitial fluid of MC3 tumour-bearing mice may induce an acceleration of MC2 growth; the increase of proteins in the interstitial fluid of MC2 tumour bearing mice may induce a slowing down of MC3 tumour growth.

Tumour growth in normal and T cell deprived mice

Groups of 10 mice were s.c. inoculated with a 1 mm^3 calibrated fragment of tumour. Each previously described tumour was grafted in 3 animal groups, syngeneic control animals, thymectomized reconstituted syngeneic animals and nu/nu mice.

The mean diameter of the tumours of each group is given in Fig. 5 as a function of the number of days after the graft. The Student's *t*-test has shown a significant slowing down for MC3 and RV2 tumour growth in T cell of deprived mice with respect to that of normal animals after a 15-day post-graft period ($0.05<P<0.001$).

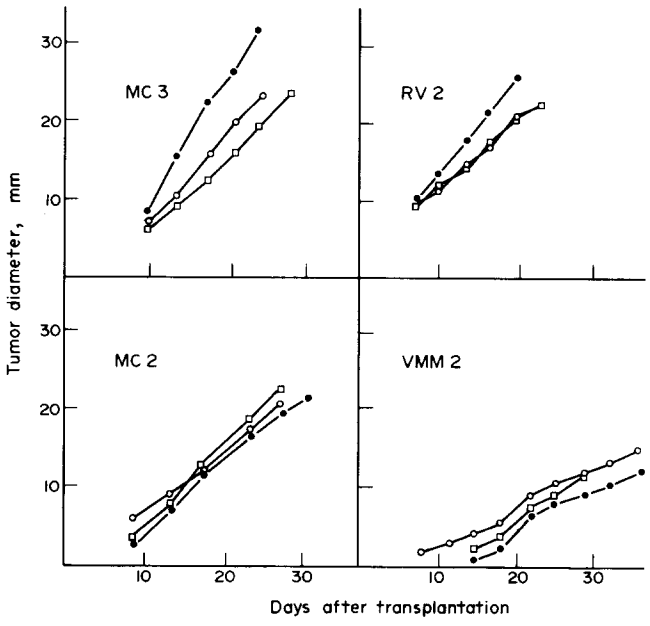

Fig. 5. Growth of MC3, RV2, MC2 and VMM2 tumours in syngeneic mice ●, thymectomized, bone marrow reconstituted syngeneic mice □, nu/nu mice ○.

The results shown in Table 2 indicate no significant variations between thymectomized-reconstituted mice and nu/nu mice bearing MC2 and MC3 tumours and on control mice.

The electrophoresis of the interstitial fluid of three control and three tumour-bearing mice was performed on the same slab. Four experiments gave similar results. Figures 6 and 7 reproduce the curves obtained after scanning of the gel slabs of interstitial fluid of control and MC2 and MC3 tumour-bearing mice. In particular, the increase observed in zones A and C for MC2 tumour in the normal system is not found in the T cell deprived system. For MC3 tumour-bearing mice, the decrease observed in zone C in the normal system is not found, but the decrease in zone A remains.

DISCUSSION

In preceding papers [1, 2], we have shown the existence of "stimulating" or "non stimulating"

Table 2. Estimation of total proteins of interstitial fluid and serum of control and tumour-bearing mice in T-cell-deprived mice according to Lowry's method

Mice	Tumour	Interstitial fluid		Serum	
CBA ♀ TXB*	—	3.15 ± 0.23†		5.07 ± 0.15	
CBA ♀ TXB	MC3	3.30 ± 0.17	NS‡	5.00 ± 0.34	NS
nu/nu ♀	—	2.83 ± 0.18		5.16 ± 0.31	
nu/nu ♀	MC3	2.48 ± 0.13	NS	4.73 ± 0.47	NS
CBA ♂ TXB	—	3.32 ± 0.07		5.23 ± 0.30	
CBA ♂ TXB	MC2	3.23 ± 0.26	NS	5.18 ± 0.19	NS
nu/nu ♂	—	3.37 ± 0.07		4.88 ± 0.40	
nu/nu ♂	MCS	3.13 ± 0.16	NS	5.18 ± 0.13	NS

*TXB Thymectomized irradiated bone–marrow reconstituted.
†Mean amounts of proteins in g/100 ml ± S.D. contained in interstitial fluid and serum of 6 control mice and 6 tumor-bearing mice (mean diameter of the tumor: 25 mm).
‡Statistical differences between the control mice group and the tumor-bearing mice group are evaluated by Student's *t*-test.
NS: not significant.

On the other hand, for the MC2 tumour, there was no significant difference of growth between normal and T-cell deprived mice, and for the VMM2 tumour there was an acceleration of growth which was significant ($P < 0.05$) in nu/nu mice.

Study of proteins in interstitial fluid and serum in T cell deprived mice (control and tumour-bearing mice)

Protein amounts in the interstitial fluid and the serum of groups of 6 mice bearing MC2 and MC3 tumours (dia 25 mm) and of the control mice have been recorded according to Lowry's method [6] in thymectomized-reconstituted CBA and nu/nu mice.

tumours on the proliferation of target cells cultured in diffusion chambers implanted s.c. near these tumours: the study of the interstitial liquid removed from near these tumours of median size, showed a decrease of proteins for the "stimulating" tumours and no variations for "non stimulating" tumours.

In this paper, we have shown that the "stimulating" tumour has a faster *in vivo* growth rate than the "non stimulating" tumour. Study of the proteins in the interstitial liquid and the serum has been extended to larger tumours. The proteic decrease found in the interstitial liquid of mice bearing the "stimulating" tumours has also been found for large tumours. However, for

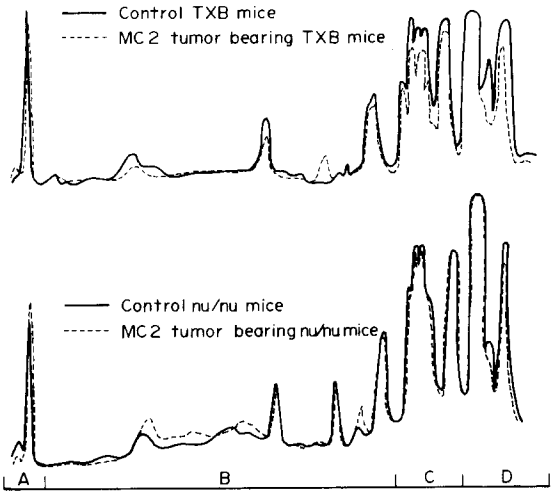

Fig. 6. Superimposed curves of polyacrylamide gradient gel electrophoresis of the interstitial fluid of control and MC2 tumour-bearing mice in TXB (thymectomized, irradiated, bone-marrow reconstituted) mice and nu/nu mice.

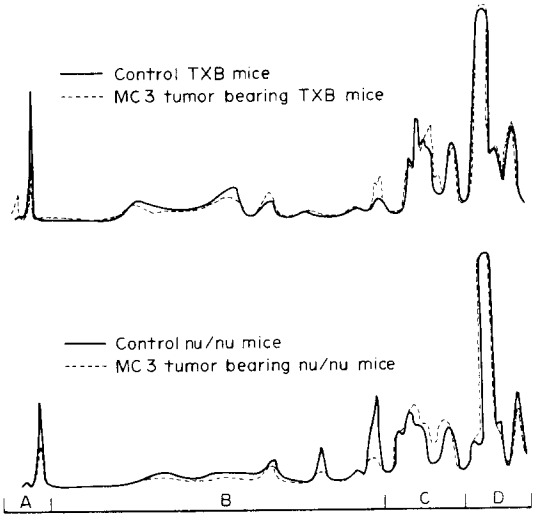

Fig. 7. Superimposed curves of polyacrylamide gradient gel electrophoresis of the interstitial fluid of control and MC3 tumour-bearing mice in TXB (thymectomized, irradiated, bone-marrow reconstituted) mice and nu/nu mice.

"non stimulating" tumours the absence of variations found for median size tumours has not been found; on the contrary a significant increase has been found in the interstitial liquid, a smaller increase was found for the serum.

The fact that the protein amount in the interstitial fluid decreases for the faster growth tumour and increases for the slower growth tumour provided an additional basis for speculation as to a possible relation between proteic variations and tumour growth rate. *In vivo*, an acceleration of the "non stimulating" tumour growth was found when it was grafted in an animal bearing a "stimulating" tumour and vice versa. This supports the hypothesis on the role of the interstitial fluid in tumour growth: indeed an interstitial fluid with less proteins would provoke an acceleration of growth; to the contrary an interstitial fluid with more proteins would provoke a slowing down of growth.

Experiments in progress show a stimulating action of the interstitial fluid of mice bearing "stimulating" tumours on the proliferation of tumour target cells in microtests.

The much more important changes noticed in the interstitial subcutaneous fluid than in the serum led us to believe that the tumour was responsible. In fact, the origin of these variations may depend either on neoplastic cells or other cells infesting the tumour.

The decrease observed in the interstitial fluid of mice bearing tumours with the faster growth may be explained simply by the nutritive requirement of some growing tumours. Also, the rapidly proliferating tissue has been shown to have a higher affinity to iron than other tissues [7]. The decrease observed for transferrin in fast growing tumours may be explained by a degradation of transferrin by tumour cells.

Different hypotheses may be made to explain the proteic increase in interstitial fluid of "non stimulating" tumour bearing mice. The simplest hypothesis may be a local increase of serum proteins due to the vascularization increase in some large tumours. Protein increase may also be explained by the synthesis of proteins by neoplasic cells. Some neoplasic human cells growing *in vitro* have been shown to synthesize ferritins [8].

The observed proteic variations may be related in part to an immunological phenomenon. Indeed, there is accumulating evidence that macrophages are numerous in tumours and that they exert a modulating effect on the tumour growth [9]. On the other hand, the presence and the quantity of lymphocytes in tumours play a role in immunostimulation or inhibition of tumour growth, the phenomenon depending on a direct lymphocyte-to-cells interaction [10, 11].

Numerous reports have shown that a variety of animals and human tumours of connective and epithelial tissue contain a large number of cells with receptors from the Fc part of immunoglobulins. These cells corresponded to infiltrating host cells, especially macrophages and antigen-activated T cells [12, 13]. Proteic increase has been observed for proteins migrating at the level of transferrin and α-globulins. Macrophages have been shown to synthesize transferrin *in vitro* [14]. Moreover, a lymphocyte growth-promoting protein indistinguishable from transferrin was isolated from human serum [15].

The high levels of α-globulins in sera from tumour-bearing hosts have been found in many instances [16–18]. These increases of α-globulins play an important role in immune tolerance. Indeed, the existence of an immunoregulatory α-globulin, which is an effective inhibitor of the immune response, has been demonstrated [19, 20]. It was hypothesized that macrophages may be the origin of this immunoregulatory substance [21].

Some works [22, 23] have shown the existence of factors produced by sensitized thymus-dependent cells which would have the property to make macrophages specifically cytotoxic for tumour cells. The molecular weight of these factors are of the same order as those of the α-globulins.

The immunological origin of these variations would be confirmed because the graft of "stimulating" and "non stimulating" tumours in T cell-deprived mice does not induce notable variations of protein amounts in interstitial fluid and serum. The hypothesis of a relationship between the proteic variations in interstitial liquid and the tumour growth is equally corroborated by the fact that tumour growth is affected in thymectomized animals and also that the proteic variations observed in a normal system no longer take place. In a normal system, for the tumour with a fast growth, the growth was accompanied by a decrease of proteins in the interstitial liquid; in an athymic system, the tumour growth was decelerated and the proteic decrease was not observed. For the tumour with a slower growth, the growth was accompanied by an increase of proteins in the normal system; in the athymic system, the tumour growth was accelerated and the proteic variation was no longer observed.

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Increased Sensitivity of Chronic Lymphocytic Leukemia Lymphocytes to Alkylating Agents due to a Deficient DNA Repair Mechanism*

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Abstract—*DNA damage was produced in suspensions of human blood lymphocytes by nitrogen mustard (HN2) and the resulting unscheduled DNA synthesis (U.S.) was determined by non-S phase labeling with tritiated thymidine. Lymphocyte samples from 20 healthy donors and 12 patients with untreated chronic lymphocytic leukemia (CLL) were tested. Maximal U.S. was induced by a 30-min exposure to 10 μM of HN2 in both cell varieties. Thymidine uptake, stimulated by this drug concentration, was higher in normal cells (270% of the control values ± 13 = S.E.M.) than in the leukemic cells (212% ± 14). In order to correlate these findings with their functional consequences normal and CLL lymphocytes pretreated with various doses of HN2 were subsequently stimulated for semi-conservative DNA synthesis with phytohemagglutinin (PHA). The dose–response curves of the HN2 induced inhibition of replicative synthesis, together with the U.S. data, suggest a deficient repair mechanism in CLL lymphocytes. The possibility that this result might be the consequence of the differing proportions of T and B lymphocytes among leukemic and normal cell suspensions was envisaged. Therefore, T-cell depleted samples of normal lymphocytes were compared to unfractionated CLL suspensions in respect to U.S. and PHA response after HN2 treatment. Results indicate that the repair deficiency observed in CLL cells is not due to the prevalence of B lymphocytes in this disease.*

INTRODUCTION

ALKYLATING agents are widely used in the treatment of lymphoproliferative diseases like chronic lymphocytic leukemia (CLL). Clinical observations suggest that in the majority of the cases of previously untreated CLL, the leukemic blood lymphocytes are particularly sensitive to several of these agents and that clinical remissions may therefore be obtained without excessive toxicity to the normal lymphocyte population. Cell sensitivity to an alkylating agent like nitrogen mustard (HN2) depends on many factors including those related to cell cycle or to drug uptake mechanisms; it also depends on the presence or absence in the cell of enzymes involved in the repair of DNA damaged by alkylation. In the particular case of normal and leukemic lymphocytes, cell sensitivity differences due to cell cycle factors can be excluded because both cell varieties represent an almost non-dividing population; furthermore no significant difference in drug uptake has been detected in these two lymphocyte varieties [1] suggesting that this particular factor may also be of little importance in this respect. The possibility therefore that differences in cell sensitivity to alkylating agents could depend on cell ability to repair damaged DNA was considered.

Repair of sublethal DNA damage produced by alkylation in mammalian cells was first suggested by Crathorn and Roberts [2] who observed that HeLa cells treated *in vitro* with varying doses of mustard gas exhibited a dose–survival curve with a shoulder suggesting that repair of alkylated DNA had occurred. De Wys and Knight [3] came to similar conclusions on the basis of the dose–response curve obtained in treating leukemia L1210 in mice with cyclophosphamide, another alkylating agent. Exposure of various mammalian cells to HN2 was further shown [4] to stimulate incorporation of labeled precursors into their DNA, as had been previously observed with u.v. light or ionizing radiations. This non-S phase labeling, representing a non-semi-conservative synthesis process, was termed unscheduled DNA synthesis and was shown to follow the excision of damaged regions

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in a DNA strand. Unscheduled DNA synthesis, which has been shown to occur also in human peripheral blood lymphocytes [5] (a practically non-dividing cell population), is generally determined by the amount of thymidine taken up by lymphocytes cultured in presence of hydroxyurea an agent known to selectively suppress semi-conservative DNA synthesis in the few dividing cells of this population [6]. In an attempt to detect eventual differences in repair ability between normal and CLL lymphocytes which could account for differences in sensitivity to HN2, we first measured unscheduled DNA synthesis in these cells after damaging *in vitro* their DNA with this drug. Furthermore, we tried to correlate these data with some parameter indicative of repair. One way of assessing the functional significance of a repair process that correlates with cell survival is to determine the ability of repaired DNA to undergo semi-conservative replication. Because most of the blood lymphocytes are normally in a resting stage semi-conservative DNA synthesis had to be stimulated with a mitogen in order to determine the proliferative response in relation to a previous alkylation.

MATERIAL AND METHODS

Lymphocyte cultures

Human peripheral blood lymphocytes were prepared under sterile conditions by the Ficoll–Metrizoate method of Böyum [7] using heparinized whole blood samples collected from 20 healthy donors and 12 patients with untreated CLL. The white blood cells in such preparations were approximately 95% lymphocytes as judged by their morphology. After two washings with Hank's TC medium the lymphocytes were suspended at a concentration of 10^6 per ml in TC 199 (Difco) buffered with 0.04 M Hepes (H-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, pH 7.6) and with 20% autologous serum. All work unless otherwise indicated was performed at 20°C.

Unscheduled DNA synthesis

One milliliter aliquots of cell suspensions were then incubated in screw capped plastic tubes with various concentrations of HN2 (Mustargen, Merck Sharp & Dohme) ranging from 1 to 10^3 μM. After 30 min the cultures were centrifuged (1000 rev/min for 10 min in a Christ Heraeus 11 KS centrifuge at 4°C) and the suspending medium replaced with fresh medium. Unscheduled DNA synthesis was determined in the lymphocyte suspensions by measuring DNA thymidine (TdR) uptake in the presence of 5 mM hydroxyurea (HU, Calbiochem). One μCi of (^{3}H) TdR (labeled in the methyl group, Radiochemical Center, Amersham, England) was added to culture samples which were then incubated for 12 hr at 37°C. The reaction was stopped by cooling the culture tubes in an ice-bath, and after extraction the radioactivity of the TCA insoluble material was determined by a standard technique [8], using a Packard Tri-carb liquid scintillation spectrometer (Model 3380). All experiments were performed on cultures in triplicate with one additional set of cultures for cell counting and viability tests as described below.

Semi-conservative DNA synthesis

HN2-damaged lymphocytes as well as undamaged controls were tested for their ability to perform semi-conservative DNA synthesis upon stimulation with a mitogen. For this purpose the cell suspensions were first treated as for unscheduled synthesis measurements and exposed during 30 min to varying concentrations of HN2 as previously mentioned except that HU and (^{3}H) TdR were omitted. After medium renewal the cells were kept at 37°C for a 12-hr period, in order to allow for DNA repair, and then cultured for 72 hr at 37°C in the presence of PHA-M (phytohemagglutinin-M Difco, 0.05 ml of the commercial solution per culture). The lymphocytes were then labeled for the last 12 hr of the culture with (^{3}H) TdR (1 μCi per culture) and the acid insoluble radioactivity was determined [8].

Cell counting and control of cell viability

Lymphocytes cultured in a special set of tubes (without PHA-M) were counted in an hemocytometer at the beginning and at the end of the incubation period. At the same time the lymphocytes were also examined for their ability to exclude Trypan blue. Cells were resuspended at a concentration of 3×10^6 per ml and two drops of culture were mixed with two drops of 0.2% Trypan blue. A total of 400 cells per series were examined and the results were expressed as a percentage of cells taking up the dye.

B-cell enrichment of normal lymphocyte suspensions

When comparing the effects of chemically induced DNA damages in lymphocytes collected from normal and from leukemic donors, the possibility that some differences may have resulted from the differing proportions of T and B cells among CLL and normal lymphocyte populations had to be considered, and adjustments were made through B-cell enrichment. For this purpose T-cell rosettes were produced in

the suspensions of normal lymphocytes by adding sheep red cells according to the method of Haegert *et al.* [9]. The cell mixture was then centrifuged on a layer of Ficoll–Metrizoate in order to sediment the rosettes and to keep the B lymphocytes at the interface layer from where they were collected washed and resuspended in TC 199 medium. The resulting cell suspensions were shown to contain from 5 to 20% rosetting cells whereas 5–28% of these cells were found in untreated suspensions of leukemic lymphocytes.

RESULTS

Unscheduled DNA synthesis

The kinetics of (^{3}H) TdR uptake by normal and CLL lymphocytes previously exposed to 5 and 10 μM HN2 concentrations and incubated in the presence of HU are presented in Fig. 1(a)

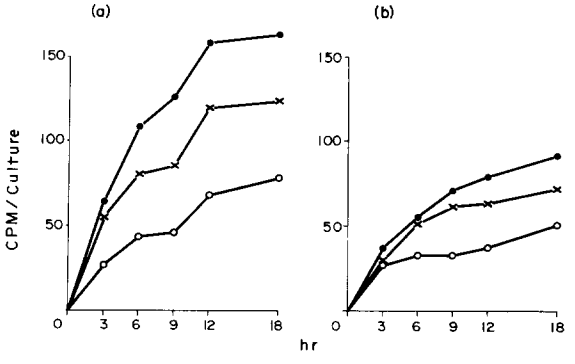

Fig. 1. The kinetics of HN2 stimulated incorporation of (^{3}H) *TdR into normal lymphocytes (a) and CLL lymphocytes (b). After a previous* 30-min *exposure to* 5 (×) *and* 10 μM *of HN2* (●) *the lymphocyte suspensions were incubated for various periods of time and the radioactivity of the TCA insoluble material determined. Control curves without HN2* (○).

(normal lymphocytes) and 1(b) (CLL lymphocytes). During the first 12 hr of incubation there is a progressive increase in DNA radioactivity with a relative flattening of the curve thereafter. The lower level of incorporation observed in unalkylated lymphocytes represents semi-conservative DNA synthesis not suppressed by HU [10, 11]. HN2 stimulated synthesis, which corresponds to the difference between these two values, remains approximately unchanged after the 12 hr-incubation so that this particular incubation time was selected for further experiments.

Dose–response curves obtained on the same lymphocyte suspensions with various concentrations of HN2 are shown in Fig. 2(a) (normal lymphocytes) and 2(b) (CLL lymphocytes). In the presence of HU, which suppresses semi-conservative DNA synthesis, there is an increasing stimulation of (^{3}H) TdR

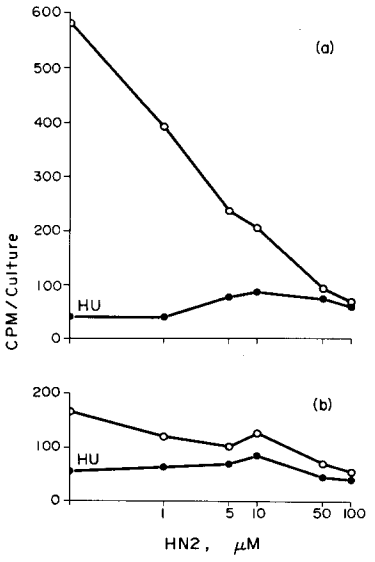

Fig. 2. (^{3}H) *TdR uptake into the DNA of normal (a) and CLL lymphocytes (b) previously exposed to increasing concentrations of HN2 and incubated for* 12 hr *thereafter. Incubation in the presence of HU* (●), *in the absence of HU* (○).

incorporation reaching its maximum in cultures preincubated with 10 μM of HN2, and followed by a decline at higher drug concentrations. When the same experiment is performed without HU there is a progressive fall in (^{3}H) TdR incorporation with increasing HN2 concentrations. This fall which occurs already in cultures preexposed to 1 μM of the damaging drug, represents selective inhibition of the semi-conservative DNA synthesis in an occasional dividing lymphocyte.

The mean values of (^{3}H)TdR uptake determined on normal and CLL lymphocytes after HN2 damage and incubation in the presence of HU, are given as percentages of control values in Fig. 3. Maximal (^{3}H) TdR uptake is again

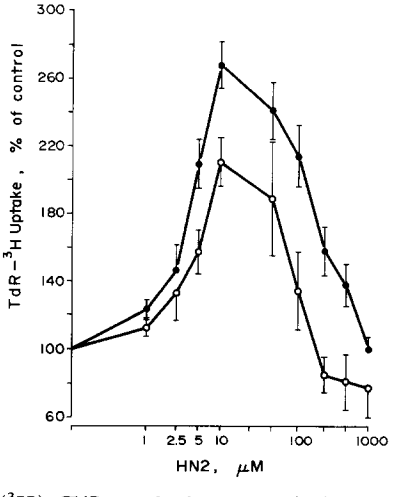

Fig. 3. (^{3}H) *TdR uptake by normal* (●) *and CLL* (○) *lymphocytes previously exposed to increasing concentrations of HN2 and incubated thereafter in the presence of HU. Results given in percentage of controls (mean values ± S.E.M.).*

observed in cultures exposed to 10 μM of HN2, but the peak values (expressed as a percentage of the control) appear to be significantly less ($2P < 0.05$) for leukemic cells (212% ± 14 = mean ± S.E.M.) than for normal lymphocytes (270% ± 13). Mean values (± S.E.M.) of (^{3}H) TdR uptake in the absence of HN2 were 55 counts/min ± 6.9 for normal lymphocytes and 40 counts/min ± 7.1 for leukemic lymphocytes, a finding which rules out the possibility that the decreased repair synthesis observed in leukemic cells could have resulted from an insufficient suppression of semi-conservative DNA synthesis by HU in these particular cells. The progressive inhibition (declining slope), which occurs at HN2 concentrations exceeding 10 μM, implies an inhibitory effect on repair synthesis which occurs at higher concentration of the drug. This inhibition which is more pronounced for CLL than for normal cells probably also affects the residual semi-conservative synthesis. Cell counts performed at the end of these experiments gave similar results in normal and leukemic cell suspensions indicating no cell loss; the Trypan blue test, however, showed a two to three times higher mortality rate among CLL cells than among normal cells (Table 1).

Table 1. *Percentage of lymphocytes unable to exclude Trypan blue after a* 30-min *exposure to HN2 followed by a* 12 hr *incubation*

HN2 (μM)	Normal lymphocytes	Leukemic lymphocytes
0	1.6 (1 – 2.5)*	3.5 (3 – 4)
1	3 (2 – 3.5)	12 (11 –13)
5	4.6 (2.5– 6.5)	15.7 (10 –26)
10	6 (4.5– 7.5)	15.2 (10 –31)
50	8.5 (6.5–11)	20.8 (9.5–34)

*Mean and extreme values.

Effect of DNA alkylation on semi-conservative synthesis in stimulated lymphocytes

The dose–response curves of HN2 pretreated lymphocytes upon PHA-M stimulation are presented in Fig. 4 which relates (^{3}H) TdR uptake to the incremental HN2 doses. Clearly at drug concentrations ranging from 0.5 to 10 μM the resulting effect is very different depending on whether normal or leukemic lymphocytes were tested. It appears that DNA synthesis is fully maintained in the former even after an incubation with 5 μM of HN2 whereas leukemic lymphocytes exhibit a definite impairment in synthesis at a ten times lower drug concentration. Thus the dose–response curve obtained with normal cells shows a shoulder which

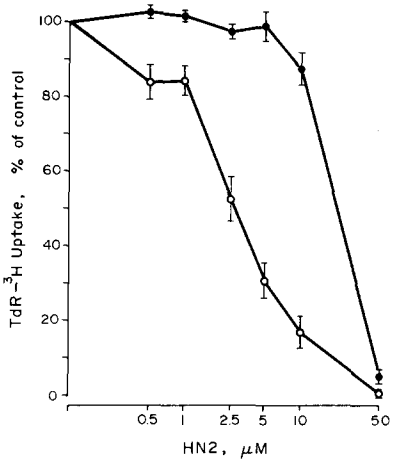

Fig. 4. *PHA induced* (^{3}H) *TdR uptake in normal* (●) *and CLL* (○) *lymphocytes previously exposed to increasing concentrations of HN2 and incubated for* 72 hr *thereafter with PHA. Results given in percentage of controls unexposed to HN2* (*mean values* ± *S.E.M.*).

is almost lacking in the case of leukemic cells. These differences could not be explained by an excessive cell loss in CLL cultures as controlled by cell counts. Furthermore cell viability averaged 74 and 65% respectively in normal and leukemic lymphocytes preexposed to a 10 μM concentration of HN2 and incubated thereafter for 72 hr.

Table 2 gives the percentages of rosetting cells determined in suspensions of lymphocytes ob-

Table 2. *Percentage of "rosette-forming" lymphocytes in various lymphocyte suspensions*

Lymphocyte suspension	Number of experiments	Rosette-forming cells (%)
Normal lymphocytes unfractionated	12	64 (53–73)*
Normal lymphocytes T-cell depleted	4	13 (5–28)
CLL lymphocytes	6	12 (5–20)

*Mean and extreme values.

tained (a) from normal donors, (b) from CLL patients and (c) in suspensions of normal lymphocytes submitted to the T-cell depleting procedure described above. The results of the PHA stimulation test performed in these various lymphocyte suspensions after previous exposure to HN2 are shown in Fig. 5. T-cell depleted and unfractionated suspensions of normal lymphocytes exhibit approximately the same type of response which differs significantly from that of the leukemic lymphocytes. Finally the ability to perform unscheduled DNA synthesis was tested in T-cell depleted and in unfractionated suspen-

sions collected from the same donors. As can be seen from Fig. 6 higher values (expressed as a percentage of the control) were observed in the first mentioned suspensions ($390 \pm 14 =$ mean $\pm$S.E.M.) than in the second ones (286 ± 20), a

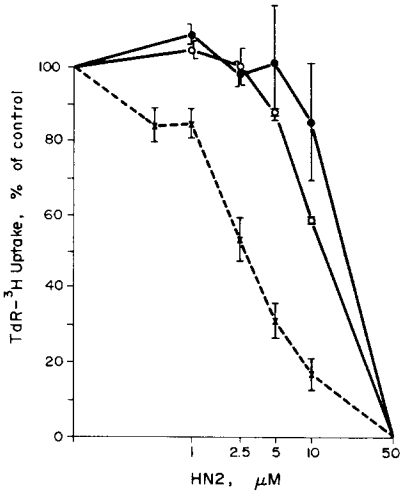

Fig. 5. PHA induced (^{3}H) TdR uptake in unfractionated suspensions of normal lymphocytes (●), T-cell depleted suspensions of normal lymphocytes (○) and unmodified suspensions of CLL lymphocytes (×). The lymphocytes were treated as in Fig. 4, and the results represent mean values ± S.E.M.

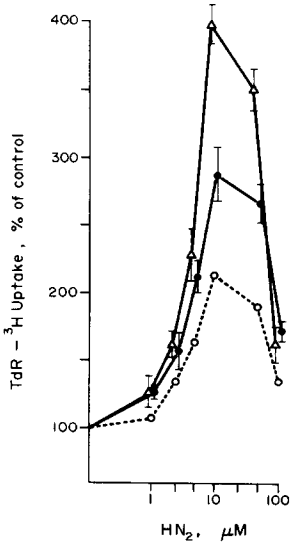

Fig. 6. HN2 stimulated (^{3}H) TdR uptake by unfractionated (●) and by T-cell depleted (△) suspensions of normal lymphocytes. The curve obtained with CLL lymphocytes and shown in Fig. 3 is indicated for comparison (○). The lymphocytes were treated as in Fig. 3 and the results represent mean values ± S.E.M.

finding which excludes the possibility of a reduced unscheduled synthesis in normal B lymphocytes.

DISCUSSION

DNA repair was first compared in normal and CLL lymphocytes by measuring unscheduled DNA synthesis following alkylation. HN2 stimulated (^{3}H) TdR uptake was found to be significantly lower in leukemic than in normal cells. This result could not be explained on the basis of thymidine pool size differences in these two varieties of lymphocytes which have been shown by others [12] not to differ in this respect. Furthermore it was also shown that the differing proportions of T and B cells in normal and leukemic lymphocyte populations could not account for the reduced level of unscheduled synthesis observed in CLL because T-cell depleted suspensions of normal lymphocytes exhibited higher unscheduled synthesis values than unfractionated suspensions from the same donor. Thus the generally reduced level of labeling observed in leukemic cells, which, nevertheless, showed a maximal (^{3}H) TdR uptake at the same HN2 concentration as normal cells, suggests a deficiency in some repair mechanism.

A second possibility of detecting differences in repair mechanisms between normal and leukemic cells was explored in looking for some biological consequence of the DNA damage, namely its ability to perform semi-conservative synthesis. This functional test was performed by stimulating the lymphocytes with PHA and observing the effect of a previous alkylation on the proliferative response. Our results show that the dose–response curves obtained in HN2 pretreated lymphocytes obtained from normal and from leukemic donors are very different: whereas DNA synthesis appears to be fully maintained up to a concentration of 5 μM of HN2 in normal cells, synthesis impairment is observed already at a 10 times lower concentration of the drug in lymphocytes collected from leukemic blood samples, a finding which suggests that functional repair is somewhat deficient in CLL. The assessment of repair ability in these two varieties of lymphocytes by comparing their response to PHA could raise some objections because of the well known observation that PHA stimulation of DNA synthesis is a weaker and slower process in leukemic than in normal cells. These differences, however, cannot explain the dissimilar behavior of these cells as observed in our system where the curves represent percentages of the control values obtained with undamaged lymphocytes. The reported data indicate only that, after incubation with given concentrations of HN2 (^{3}H) TdR uptake is relatively more depressed in lymphocytes obtained from leukemic than from normal blood.

As the PHA response may also depend on the percentage of T lymphocytes in a cell pop-

ulation, the HN2 stimulated repair process of leukemic cells was compared to that of a population of normal lymphocytes in which the proportion of T cells had been lowered to the level observed in leukemic samples. The results obtained with both normal and leukemic cell suspensions containing less than 20% rosetting lymphocytes showed that the T-cell depleted normal lymphocyte samples behaved after alkylation like unfractionated normal lymphocytes when stimulated with PHA.

Thus, even after T-cell adjustment, PHA stimulated semi-conservative DNA synthesis appears to be very much more sensitive to alkylation in the case of CLL than in normal controls. These results however have to be interpreted with caution because PHA is generally considered to be a specific T-cell stimulant [13]. If only T cells were activated by this mitogen then our data would imply a T-lymphocyte deficiency in patients with CLL, a disease known however to involve B lymphocytes. If on the contrary one admits, as proposed by several authors [14, 15] that leukemic B-cells may also participate in the PHA stimulation process, then a deficient repair mechanism in these particular cells is conceivable. Activation of leukemic lymphocytes is indeed suggested by DNA synthesis studies in cell suspensions cultured with PHA for periods longer than 3 days: whereas normal controls show a peak response after 3 days and reduced values of (^{3}H) TdR uptake after 5 days, maximal response occurs between the 5th and the 7th day in the case of CLL.

Since normal lymphocytes synthesize less DNA after a 5-day incubation period, it appears that those cells which take up increasing amounts of (^{3}H) TdR after a 5-day incubation period are mainly the leukemic ones [14, 15]. We have confirmed these data on 3 and 5 day-cultures of normal and leukemic lymphocyte suspensions stimulated with PHA (results not shown). Furthermore, when the effect of a previous alkylation was examined on these 5-day cultures the same difference between the normal and the leukemic dose–response curves as reported in 3-day cultures, was again observed. This finding as well as the reduced level of unscheduled DNA synthesis demonstrated in leukemic lymphocytes following an exposure to HN2 suggests that these cells are deficient in some DNA repair mechanism.

Further studies will be needed to confirm these observations by more direct techniques designed to detect the nature of the differences in DNA fragmentation and repair processes occurring in normal and leukemic lymphocytes.

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Studies on the Effects of the Mammary Carcinogen 7,12-Dimethylbenz(a)anthracene on Release and Synthesis of Prolactin by Rat Pituitaries *In Vitro**

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Abstract — *These* in vitro *experiments have shown that 7,12-dimethylbenz(a)anthracene (DMBA) at concentrations of* 5 μg *and* 50 μg/ml *can inhibit release of prolactin by female rat pituitaries and that the pituitary at pro-oestrus is more sensitive than at other stages of the oestrous cycle. The use of specific adrenergic blocking agents has shown that the inhibitory reaction occurs through binding of the carcinogen at α- and β-receptor sites. A chemically related non-carcinogenic hydrocarbon phenanthrene, did not inhibit prolactin release. Synthesis of prolactin was inhibited by the higher concentration of DMBA but the adrenergic blocking agents did not ameliorate this effect. The release and synthesis of prolactin by male rat pituitaries was also inhibited by DMBA. The release of growth hormone by these pituitaries* in vitro *was not inhibited by the carcinogen.*

INTRODUCTION

THE ROLE of prolactin in 7,12-dimethylbenz(a)-anthracene (DMBA) induced mammary cancer in the rat has been shown to be a complex one. Clemens *et al.* [1] showed that lesions of the median eminence prior to a single intravenous injection of DMBA significantly reduced tumour incidence whereas similar lesions made at 75 days after DMBA administration stimulated tumour growth. Release of prolactin is known to be mainly under inhibitory hypothalamic control, probably by dopamine [2], and lesions in the hypothalamus would presumably destroy the inhibitory effects. It would appear then that increased prolactin release at the time of carcinogen administration is inhibitory of tumour induction but that prolactin stimulates growth of established tumours.

It has been reported that following a single intravenous injection of DMBA in female Sprague–Dawley rats there was a massive release of prolactin into the plasma which was not followed by compensatory hormone synthesis [3]. It would seem that the carcinogen may inhibit release of a prolactin inhibitory factor (PIF) by acting on the hypothalamus, but it is also possible that the carcinogen can directly affect synthesis and release of prolactin by the pituitary. To see if this was happening a series of experiments has been made on rat pituitaries *in vitro* to determine if DMBA could have a direct and specific effect on prolactin release and synthesis.

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MATERIAL AND METHODS

The animals used in these experiments were eight week old female Wistar rats bred in the Zoology Department, Sheffield University. Rats were killed by decapitation and the pituitary immediately removed and placed in a drop of TC199 culture medium. Each pituitary was bisected sagittally, one half serving as the control for its opposite half. Hemi-pituitaries were pre-incubated for 30 min in 200 μl TC199 at 37°C. After pre-incubation each half pituitary was incubated for 5 hr at 37°C in small tubes held in a Dubnoff shaker with a constant atmosphere of 95% O_2 in CO_2. Each tube contained 100 μl TC199 with 10 μCi/ml 4.5 ^{3}H-leucine so that hormone synthesis as well as release could be measured. Appropriate drugs were added to both pre-incubation and incubation media. Prolactin and growth hormone content of both pituitary and medium were measured by densitometry after polyacrylamide gel elec-

trophoresis (PAGE), at the end of the incubation period.

Polyacrylamide gel electrophoresis (PAGE)

The system of Davis [4] was used to separate the hormones except that the samples were not incorporated into gels. Identification of rat growth hormone and prolactin after separation by this system has been made by Jones *et al.* [5] and Yanai and Nagasawa [6]. At the end of the incubation period the total volume of medium was layered onto a single gel, the pituitary weighed and homogenised in Tris/glycine buffer pH 8.3. After solubilisation in ice for 30 min the equivalent of 0.25 mg wet weight of pituitary was layered onto a gel. At the end of the electrophoresis run gels were stained overnight in Coomassie Blue (0.1% in water:methanol:acetic acid, 8:1:1) and destained in the dye solvent until the background cleared.

The amount of hormone in each stained band was quantified by densitometry using a Joyce-Loebl "Chromoscan". A standard curve for rat prolactin run on similar gels was constructed by running various amounts of rat prolactin, in duplicate, and staining the gels with Coomassie Blue (Fig. 1). Thus experimental results could be expressed as μg prolactin. Radioactivity in the separated hormones was measured by the method of Tishler and Epstein [7], a piece of gel from beyond the ion front was used as background control.

For the sake of clarity the procedure for each experiment will be described separately together with the results.

1. *Action of DMBA on release of prolactin by female rat pituitaries*

DMBA (Light & Co.) was recrystallised from ethanol and tested for its effect on release and synthesis of prolactin by female rat pituitaries *in vitro* as follows. The carcinogen was dissolved in absolute ethanol and added to the incubation medium at three dose levels, 50 μg/ml, 5 μg/ml and 0.5 μg/ml, each time dissolved in 10 μl ethanol. Ten microlitres of ethanol was added to the control hemipituitaries.

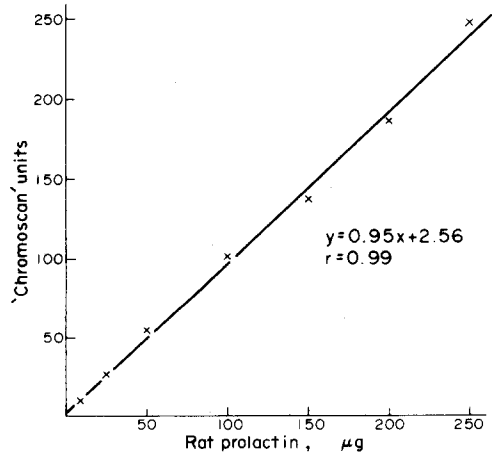

Fig. 1. Standard curve for the estimation of rat prolactin stained with Coomassie blue after polyacrylamide gel electrophoresis. Measurements were made by densitometry on a Joyce-Loebl "Chromoscan".

The results of these experiments are shown in Fig. 2, in which the amount of hormone in the medium is expressed as μg/mg pituitary. It can be seen that there was a significant reduction in the release of prolactin in the presence of 50 μg and 5 μg/ml DMBA but that 0.5 μg/ml had no significant effect (Student's *t*-test). There was also a significant reduction in the release of hormone by male rate pituitaries as shown in the final pair of columns in Fig. 2.

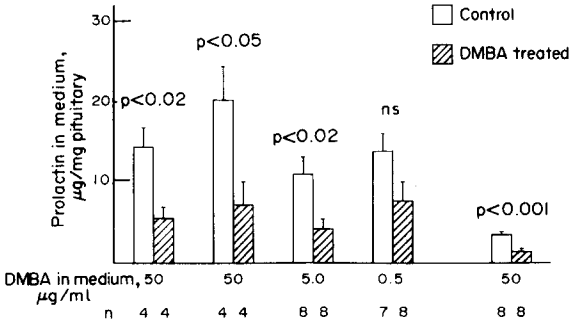

Fig. 2. Effect of various concentrations of DMBA on the release of prolactin by rat pituitaries in vitro. *The first four pairs of columns are female and the final pair are male rat pituitaries.*

These results showed that DMBA could inhibit release of prolactin *in vitro*. To see if this action was specific and not simply a toxic reaction several experiments were performed as follows.

2. *Action of adrenergic blocking agents on DMBA-mediated inhibition of prolactin release*

It has been shown by Shaar and Clemens [2] in an elegant series of experiments that dopamine is the agent in hypothalamic extracts which inhibits prolactin release by rat pituitaries *in vitro*, suggesting that this is probably the *in vivo* inhibitory agent. MacLeod and Lehmeyer [8] have demonstrated by the use of specific blocking agents in an *in vitro* system that dopamine acts via the α-adrenergic receptor and the dopamine receptor site. These experiments have formed the basis for examining the specificity of DMBA-mediated inhibition of prolactin release. All the blocking agents were made up in 0.01 N HCl containing 10 μg/ml ascorbic acid and added to the incubation medium as 10 μl

aliquots to give the final desired concentration. All controls received the solvent only.

(a) *Haloperidol (Serenace, G. D. Searle & Co. Ltd.).* Haloperidol has been shown to block the dopamine receptor in pituitary prolactin cells [8].

Control pituitary halves were treated with 5 μg/ml DMBA whilst their opposite halves were incubated with 5 μg/ml DMBA together with 0.7 μg/ml haloperidol. It can be seen from the results (Fig. 3) that haloperidol did not significantly increase the release of prolactin in the presence of DMBA. Therefore the carcinogen is not acting via the dopamine receptor.

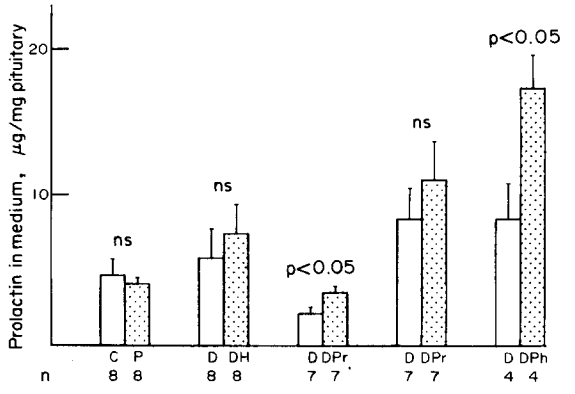

Fig. 3. Results of experiments on the effects of various drugs on the specificity of DMBA-mediated inhibition of prolactin release. C.-ethanol only (10 μl/min). *P.-phenanthrene* (35 μg/ml). *D.-DMBA* (50 μg/ml). *DH.-DMBA* (5 μg/ml) + *haloperidol* (0.7 μg/ml), *DPr.-DMBA* (50 μg/ml) + *propranolol* (4×10^{-5} M), *DPh.-DMBA* (50 μg/ml) + *phentolamine* (4×10^{-5} M).

(b) *Phentolamine (Phentolamine mesylate. CIBA).* Phentolamine, which is an α-adrenergic receptor blocking agent, was tested for its effect on DMBA-mediated inhibition of prolactin release by adding 14.9 μg/ml (4×10^{-5} M) phentolamine to incubations containing 50 μg/ml DMBA. The result in Fig. 3 shows that phentolamine blocked the action of the carcinogen and significantly increased the release of prolactin. DMBA is therefore acting as the α-adrenergic receptor.

(c) *Propranolol (D-L propranolol hydrochloride. Sigma).* Four times 10^{-5} M concentration of propranolol significantly increased the release of prolactin by rat pituitaries incubated with 50 μg/ml DMBA as compared with controls treated with DMBA alone in one experiment but not in a second (Fig. 3). However, the prolactin in the medium expressed as a percentage of the total in medium plus pituitary combined did give a significant result in both experiments (Table 1). This shows that the carcinogen may act via the β-adrenergic receptor site as well as the α receptor, but as propranolol may have slight blocking effects at the α receptor too this could contribute to the reduced inhibition by DMBA.

Table 1. Effect of specific blocking agents on 7,12-dimethylbenz(a)anthracene (DMBA) mediated inhibition of prolactin release by rat pituitaries in vitro

Treatment	*n*	Prolactin in medium $\frac{M \times 100}{M+P}$
DMBA (5 μg/ml)	7	7.13 ± 2.07 NS
DMBA (5 μg/ml) + Haloperidol (0.7 μg/ml)	7	10.48 ± 2.62
DMBA (50 μg/ml)	8	3.40 ± 1.04 $P<0.05$
DMBA (50 μg/ml) + Propranolol (4×10^{-5} M)	6	7.10 ± 1.21
DMBA (50 μg/ml)	8	6.07 ± 1.32 $P<0.05$
DMBA (50 μg/ml) + Propranolol (4×10^{-5} M)	8	14.36 ± 2.07
DMBA (50 μg/ml)	4	5.5 ± 1.27 $P<0.01$
DMBA (50 μg/ml) + Phentolamine (4×10^{-5} M)	4	14.67 ± 2.07

3. *Effect of a related non-carcinogenic hydrocarbon on release of prolactin by rat pituitaries*

To test if the inhibition of prolactin release by DMBA had any significance for the carcinogenic potency of DMBA a structurally related non-carcinogenic hydrocarbon phenanthrene was tested in the *in vitro* system. Phenanthrene was recrystallised from ethanol and added to the incubation medium to give a final concentration of 35 μg/ml (2×10^{-4} M), equimolar with 50 μg/ml DMBA. Phenanthrene did not inhibit prolactin release (Fig. 3).

4. *Effect of DMBA on release of growth hormone by rat pituitaries* in vitro

During the experiments the release of growth hormone was also measured to see if the action of the carcinogen was specific to prolactin. It can be seen in Table 2 that none of the three dose levels of DMBA used had a significant effect on the release of growth hormone, indicating that the carcinogen had a specific interaction with the prolactin cells.

5. *Oestrous cycle and inhibition of prolactin release by DMBA*

In a number of these experiments the results were variable between individual pituitaries

Table 2. Effect of 7,12-dimethylbenz(a)anthracene (DMBA) on release of growth hormone by rat pituitaries in vitro

Treatment	*n*	Growth hormone in medium* $\frac{M \times 100}{M+P}$
Control (Ethanol only)	7	3.17 ± 0.46 NS
DMBA (50 μg/ml) (2×10^{-4}M)	8	3.67 ± 0.91
Control (Ethanol only)	8	3.67 ± 0.62 NS
DMBA (5 μg/ml) (2×10^{-5}M)	8	5.87 ± 1.55
Control (Ethanol only)	6	3.39 ± 0.51 NS
DMBA (0.5 μg/ml) (2×10^{-6}M)	8	2.90 ± 0.57

*Chromoscan readings.

leading to large standard errors. The secretion of prolactin by rat pituitaries varies during the normal oestrous cycle and both pituitary prolactin content and *in vitro* secretion are higher during pro-oestrous and oestrus than dioestrus [9]. To see if the stage of the oestrous cycle had any effect on the degree of prolactin inhibition by DMBA the following experiment was performed.

Vaginal smears were taken from 8 week old rats and groups of 4 in each stage of proestrus, oestrus, metoestrus or dioestrus were used to measure the effect of 50 μg/ml DMBA on prolactin release as previously described. The results are shown in Fig. 4 in which it can be seen that there is variation in the degree of inhibition of prolactin release at different stages of the cycle with the least inhibition at dioestrus. Figure 5 shows that the amount of prolactin in the medium of the DMBA treated pituitary halves as a percentage of the controls was lowest at pro-oestrus. This indicates that the pituitary at pro-oestrus is most sensitive to DMBA-mediated inhibition of prolactin release.

6. *Effect of various drugs on rat prolactin* in vitro

The various drugs added to the incubation media may have had a direct effect of preserving or destroying prolactin in the medium or of interfering with the electrophoretic separation of the hormones. To check this possibility pituitaries from 13 young mature female rats were each cut into three parts and incubated in 4.5 ml TC199 at 37°C for 2 hr. The pituitaries and loose cells were spun down and the supernatant collected. Eight 0.5 ml aliquots were taken and

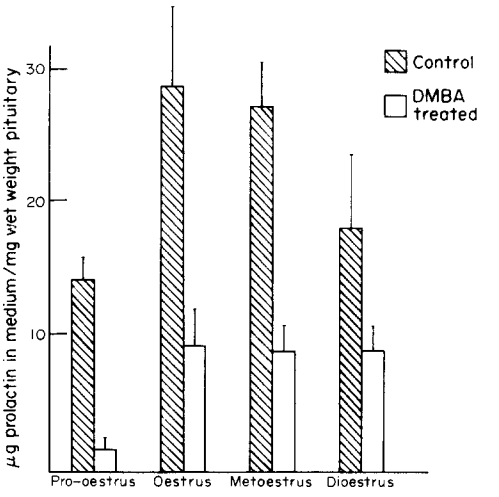

Fig. 4. Inhibitory action of DMBA (50 μg/ml) on prolactin release by female rat pituitaries at different stages of the oestrous cycle.

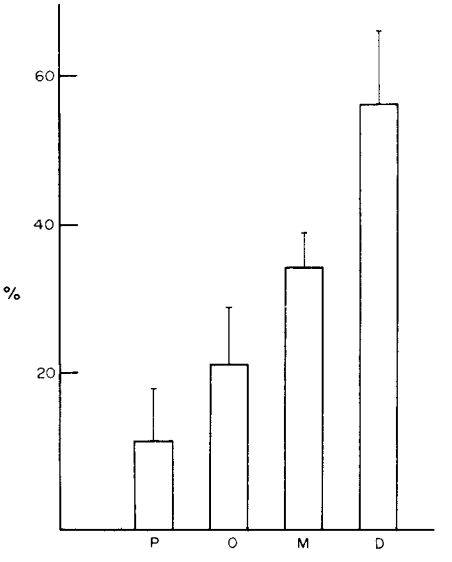

Fig. 5. Prolactin in the medium of DMBA (50 μg/ml) treated hemipituitaries as a percentage of that secreted by their control hemipituitaries.

to an aliquot was added one of the following drugs: (a) phentolamine (4×10^{-5}M) (b) propranolol (4×10^{-5}M) (c) haloperidol (0.7 μg/ml) (d) DMBA (50 μg/ml) (e) ethanol (10 μl/ml) (f) ascorbic acid in 0.01N HCl (10 μg/ml), one aliquot received no additions and a further one was not incubated. Thus all but one of the aliquots contained one of the drugs used in the incubation experiments at the specified concentration.

Four 100 μl samples were taken from each of the aliquots and incubated separately for a further 4 hr at 37°C. It can be seen from Table 3 that there was no significant difference in the prolactin content of any of the incubated tubes whether or not a drug was added. The experiment does demonstrate however that there is

Table 3. Effect of various drugs on rat prolactin secreted in vitro

Treatment	*n*	μg Prolactin per 100 μl incubation medium
Phentolamine (4×10^{-5}M	4	22.31 ± 1.0
Propranolol (4×10^{-5}M)	3	24.33 ± 2.81
Haloperidol (0.7 μg/ml)	4	22.83 ± 0.90
DMBA (50 μg/ml)	4	21.52 ± 1.77
Ethanol (10 μl/ml)	4	23.10 ± 1.10
Ascorbic acid (10 μg/ml in 0.01 N HCl)	4	23.10 ± 0.30
No additions	4	22.57 ± 0.61
No additions + No incubation	3	29.94 ± 0.61

some loss of prolactin during incubation and shows that readings of prolactin content in media after several hours incubation represents a balance between secretion and breakdown and therefore the apparent rate of secretion is an underestimate.

7. *Effect of DMBA on synthesis of prolactin by rat pituitaries* (in vitro)

Synthesis of prolactin by both male and female rat pituitaries was significantly reduced by 50 μg/ml DMBA (Table 4). The inhibition of synthesis however was not ameliorated by either phentolamine or propranolol (Table 4), suggesting that inhibition of synthesis is caused by interaction of the carcinogen at intracellular sites different from the adrenergic receptors.

Table 4. Effect of 7,12-dimethylbenz(a)anthracene (DMBA) on 4.5 ^{3}H-leucine incorporation into rat prolactin in vitro *and its modification by adrenergic blocking agents*

Treatment	*n*	Newly-synthesised prolactin in medium + pituitary (counts/min per mg pituitary)
Control (Ethanol only)	8	3340.8 ± 1085.2
		$P<0.05$
DMBA (50 μg/ml)	8	1110.2 ± 235.8
DMBA (50 μg/ml)	8	1364.5 ± 445.8
		NS
DMBA (50 μg/ml) + Propranolol (4×10^{-5}M)	6	990.2 ± 339.5
DMBA (50 μg/ml)	8	1195.9 ± 297.3
		NS
DMBA (50 μg/ml) + Phentolamine (4×10^{-5}M)	7	651.2 ± 124.1
Male rat pituitaries		
Control (Ethanol only)	8	246.6 ± 28.13
		$P<0.001$
DMBA (50 μg/ml)	8	89.0 ± 15.3

DISCUSSION

Support for the view that the stimulated rat mammary gland is refractory to tumour development has been provided by a number of different experiments. Dao and Sunderland [10] observed that pregnant rats treated with the carcinogenic hydrocarbon 3-methylcholanthrene (3-MC) failed to develop mammary tumour and Dao, Bock and Greiner [11] showed that lactating rats were also refractory to tumour induction by 3-MC. Treatments known to raise the circulating levels of prolactin, lesions in the median eminence [1, 12, 13], administration of reserpine [14], combined treatment with norethynodrel and mestranol [15] and grafting of additional pituitaries beneath the kidney capsule [16] all reduced the incidence of mammary tumours in rats subsequently treated with DMBA. Thus if DMBA can inhibit release of prolactin, as demonstrated in these *in vitro* experiments, it can clearly enhance its own ability to induce mammary tumours. It may be that a temporary reduction in circulating prolactin leaves binding sites free in the mammary gland with which DMBA may interact. However Dao and Sinha [3] showed that within a few hours there was a massive increase in circulating levels of prolactin after a single intravenous injection of DMBA, which would stimulate the mammary gland. In contrast to these results recent experiments by Valero *et al.* [17] have shown that up to 30 hr after intragastric instillation of DMBA into female rats there is a reduced level of plasma prolactin when given at any stage of the oestrous cycle together with elevated pituitary prolactin content. The divergence of these results may be due to the different routes of administration of the carcinogen and different dose levels used, nevertheless both treatments are commonly used to induce mammary tumours and clearly much more investigation is needed.

The results of Dao and Sinha [3] also suggest that the carcinogen acts at the hypothalamic level as well as the pituitary to produce opposite effects, one to increase and the other to decrease the release of prolactin. Not only does DMBA act at these sites and the mammary gland it also causes considerable damage to the adrenal

cortex shortly after injection [18] which may influence metabolism of the carcinogen and prolong its presence in the body. Thus DMBA administered systemically has many actions in various tissues some or all of which may influence the ultimate development of mammary tumours.

In these *in vitro* experiments the concentration of carcinogen used in the incubation medium is comparable with or less than those used *in vivo* to induce mammary tumours. Huggins *et al.* [19] used various doses of DMBA to induce mammary tumours of which 1, 2.5 and 5 mg per rat given intravenously produced tumours in 80–100% of the animals without toxic effects. The average weight of such rats is about 140 g and if the blood represents 8% of body weight then the lowest dose of 1 mg would give a concentration of 89.2 μg/ml. The highest dose, 5 mg, is the most commonly used one for mammary tumour induction and therefore gives a much higher blood concentration.

The inhibition of prolactin synthesis by DMBA *in vitro* would explain the *in vivo* observations of Dao and Sinha [3] that prolactin secreted from the pituitary during the first few hours after a single intravenous injection of DMBA was not replaced, but these results again conflict with those of Valero *et al.* [17] who found an increase in pituitary prolactin content. The *in vitro* inhibition did not appear to be ameliorated by either phentolamine or propranolol since there was no significant difference between the control and test groups. The absence of a blocking effect suggests that DMBA may affect prolactin synthesis via a different mechanism from that concerned in inhibition of release. DMBA can combine with DNA and RNA as well as protein [20, 21] and it may be that such an interaction causes reduced synthesis of prolactin. To clarify this and reduce the variability between pituitaries, experiments with pools of dispersed cells would be most valuable. Yanai and Nagasawa [22] noted that in female rats bearing DMBA-induced mammary tumours the pituitary content and concentration of both growth hormone and prolactin were significantly lower than in controls. This could be due to the actively growing tumours or to a permanent effect of the single instillation of the carcinogen.

Mammary cancer can be induced in a low percentage of male rats by a pulse dose of DMBA [23], but Dao and Greiner [24] were able to induce mammary tumours in male rats only after ovarian implantation. This shows that oestrogenic hormones may be important in tumour initiation. It has been shown recently that oestrogen administered *in vivo* to lactating rats can inhibit prolactin binding to specific receptors in the mammary gland [25] which again suggests that the effect of oestrogens in mammary cancer induction may be related to reduced prolactin binding in the mammary gland at the initiation stage.

The action of DMBA at the α and possibly the β-receptor sites differs from that of dopamine which has been shown to inhibit prolactin release via the α-receptor and the dopamine receptor [8]. However in a teleost fish (*Poecilia latipinna*) dopamine appears to inhibit prolactin release by interaction at both adrenergic receptor sites as well as the dopamine receptor [26]. The nature of adrenergic receptors in pituitary cells is unknown but Cuatrecasas *et al.* [27] using labelled catecholamines has shown binding to membrane proteins in a variety of tissues in the rat. What determines the site of binding is however not clear but it has been suggested [27] that it may be governed by the presence of the catechol-o-methyl transferase enzyme. The pathway by which the adrenergic agents ultimately produce their effects is still not known.

The results of these *in vitro* experiments show that more studies must be made on pituitary hormone synthesis and release and on mammary tissue receptors during the first few hours after carcinogen administration *in vivo*. More work must also be done with pituitary tissue *in vitro* for prolonged periods to determine the longer-term effects of the carcinogenic hydrocarbons.

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Perspectives in Cancer Research

Hemostatic Abnormalities Associated with Malignant Diseases*

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I. INTRODUCTION

IMPROVEMENTS in early diagnosis and supportive care are major problems in cancer research. Progress achieved in oncological surgery and radiotherapy as well as improved chemotherapy programs can significantly alter the natural history and have led to an increasingly better prognosis for patients with malignant diseases. Morbidity and mortality is in many cases determined not only by the primary disease but also by overwhelming fatal or non-fatal complications. Beside infections, thromboembolic and hemorrhagic phenomena are of significant importance. During the last decade characteristic laboratory and morphological data could be correlated to the clinical symptoms. This paper is intended to give a summary of the present knowledge and to demonstrate possibilities for their diagnostic and therapeutic use in clinical practice.

II. CLINICAL SIGNS

Thromboembolic complications such as venous thromboses, phlegmasia coerulea et alba dolens, thrombophlebitis migrans and pulmonary embolism as well as hemorrhagic diathesis and paradoxically sometimes both of them, are often primary symptoms and typical complications in the course of neoplastic diseases. The incidence in solid tumors varies between 10 and 30% [1–4]. In particular malignant adeno-carcinomas are associated with non-bacterial thrombotic endocarditis appearing in about 1–5% of all cases [5–6]. Vascular accidents can be detected in about 50% of patients with polycythemia vera [7]. In acute promyelocytic leukemia wide-spread intravascular coagulation involving the microcirculation and combined with a severe bleeding tendency is a very common finding [8–10]. In contrast, most malignant hematological diseases associated with thrombocytopenia are characterised in their history by a generalised tendency to bleed. Interestingly enough, however, even in these cases thromboembolic complications, fulminant pulmonary embolism and cardiac infarctions can occur [11–13].

Statistical data concerning the causes of death in patients with cancer [14–16] and malignant hematological diseases [17–19] demonstrate the high incidence of fatal bacterial infections. Next to these complications thromboembolic events are the main cause of death in solid tumors, while in malignant blood diseases a generalised bleeding tendency is a predominant finding at autopsy.

III. LABORATORY FINDINGS

In systematic studies hemostatic abnormalities are detectable in about 95% of patients with malignancies, with or without there being any clinical evidence of a thromboembolic or hemorrhagic tendency. According to Sun *et al.* [20] 82% of the cases under investigation had increased fibrin degradation products, 57% had prolonged prothrombin time, 55% had hyperfibrinogenemia, 55% had a positive ethanol gelation test indicating elevated levels of fibrin monomers, 47% had prolonged thrombin time and 28% had prolonged or shortened partial thromboplastin time. Thrombocytopenia was present in only 30% and hypo-fibrinogenemia in 11%. In a series of 50 patients with inoperable lung cancer [21] fibrinogen was increased in 82%, prothrombin time was prolonged in 62%, fibrin split products were increased in 38%, thrombocytosis was present in 30% and the ethanol-gelation test was positive in 11% of the

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cases. A reduced activity of factor XIII and defects of fibrin stabilization can be detected in about 65% of the patients with acute leukemia at the time of hospitalisation [22]. In this chapter typical results in various forms of malignant diseases will be considered in detail.

1. *Platelets*

An elevated platelet count is a characteristic finding in myeloproliferative disorders. In addition, in polycythemia vera there is an increase in blood viscosity due to erythrocytosis. A nonspecific, and in the first instance inexplicable rise in the platelet count, is to be seen in the initial phase and in the course of several solid tumors and malignant lymphomas [23–27]. It should be mentioned that in a small number of cases of acute leukemia an enhanced proliferation of megakaryocytes (Fig. 1) and thrombocytosis occur [28, 29]. However, thrombocytopenia is the characteristic symptom in patients with acute leukemia. It can also appear in the course of other malignant diseases in which cases it is necessary to examine whether it is due to neoplastic bone marrow infiltration, bone marrow depression following cytostatic chemo- or radiotherapy or enhanced peripheral consumption. It should be mentioned that idiopathic thrombocytopenic purpura complicating chronic lymphocytic leukemia was described [30]. Impairment of platelet function, particularly disturbances of ADP- and collagen-induced platelet aggregation, have been demonstrated in patients with preleukemia, acute leukemia, myeloproliferative diseases and dysproteinemias [31–39]. The cause and mechanism of the platelet defect are not completely understood.

2. *General tests of hemostatic function*

Prolonged bleeding time is to be found in patients with thrombocytopenia and/or platelet dysfunction. In about 50% of patients with solid tumors there is a significant shortening of whole blood clotting time, a highly significant reduction of plasma recalcification time, a shortening of siliconised glass clotting time, decreased partial thromboplastin time, accelerated thromboplastin generation and a decreased heparin tolerance, while prothrombin time and thrombin time are slightly prolonged [40–45].

Surprisingly, the tendency of the blood to clot is distinctly higher also in patients with acute leukemia compared to normal control groups [46–49]. In 1952 Tagnon *et al.* [50] described enhanced blood fibrinolytic activity in patients suffering from metastatic cancer of the prostate. Studies involving general tests of the fibrinolytic enzyme system (whole blood clot lysis test, plasma dilution clot lysis test, euglobulin clot lysis test) which have been carried out in a systematic way in other types of malignancy gave controversy results and have demonstrated clinically relevant enhanced fibrinolysis in only a few cases [51–57]. Widespread pathological changes of hemostatic functions as have been shown by means of such tests as those quoted above are very common in cases of malignant diseases with severe liver dysfunction and with disseminated intravascular coagulation.

3. *Determination of clotting factors*

Patients suffering from malignant diseases, without any complications, regularly exhibit an increased activity in the plasmatic blood clotting factors. The fibrinogen level is clearly raised in about 30–50% of the cases with solid tumors as well as in patients with acute leukemia [2, 41, 49, 58]. Among the clotting factors II–XII the most marked increases in activity can be seen in factor VIII/anti-hemophilic globulin [59–61]. The immunologically identified antigen associated with factor VIII is elevated also (Fig. 2).

In contrast to the clotting factors quoted above the fibrin stabilizing factor (blood clotting factor XIII, plasma transglutaminase) frequently has a reduced biological activity, particularly in patients with acute leukemia [22] and in disseminated cancer [62, 63]. There are contradictory results concerning the determination of fibrinolytic factors. Ogston *et al.* [54] found reduced plasminogen levels in 14 out of 43 patients with leukemia. Studying a group of 100 unselected patients with disseminated malignancies Soong and Miller [56] found plasminogen levels within the normal range. In a series of 179 unselected cancer patients Thornes *et al.* [64] found a definite increase in the anti-plasmin levels. The mean value for the inhibitors was approximately twice that of the normal.

In a few cases of patients with acute leukemia an isolated and clinically relevant decrease in the blood clotting activity of factors V and X has been described, without, however, any evidence of the participation of other clotting factors being mentioned. The pathogenesis of this abnormality is not clear [65]. Acquired factor VIII inhibitors have been described in a few patients with malignant lymphomas [66] and dysproteinemias [67]. In macroglobulinemia the most frequently observed inhibitor is one that interferes with fibrin monomer aggregation [68, 69]. Dysfibrinogenemia was described in some patients with primary hepatoma [70, 71].

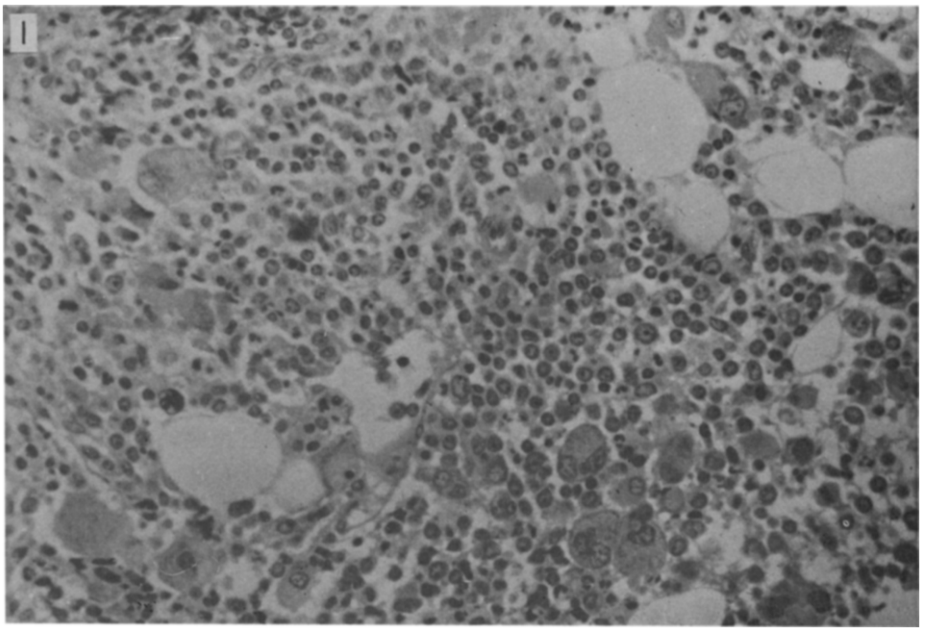

Fig. 1. Unusual case of acute myeloblastic leukemia with high number of megacaryocytes, abnormal "microcaryocytes" and thrombocytosis (1·6 million platelets/μl blood).

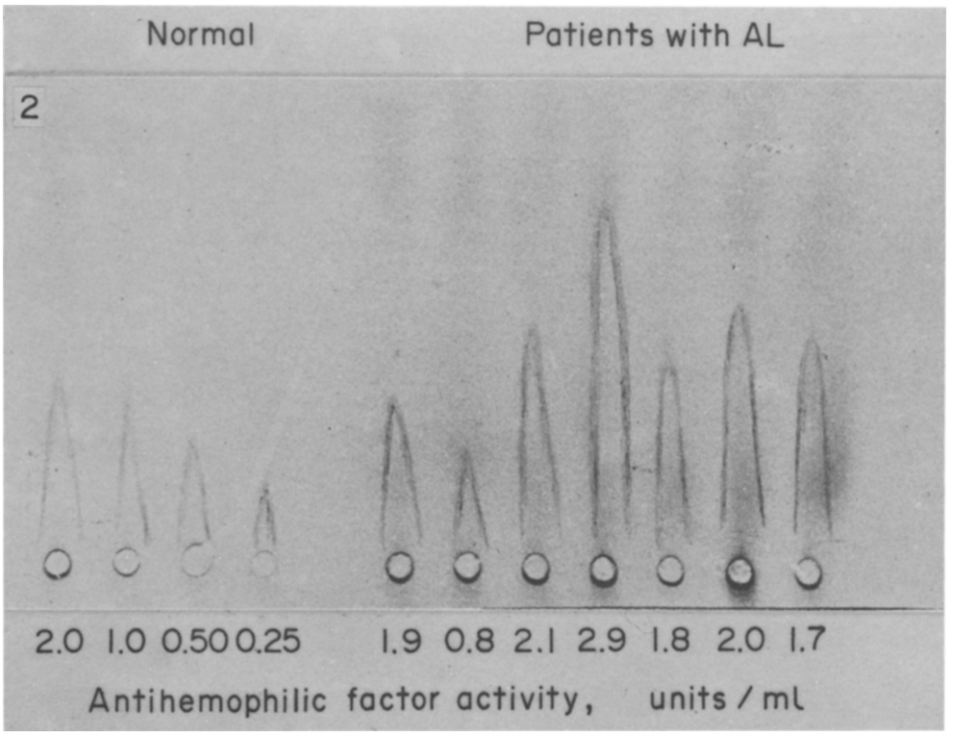

Fig. 2. Clotting factor VIII *activity (units/ml) and factor* VIII *associated antigen (immuno diffusion electrophoresis) in seven patients with acute leukemia (AL). As a control a normal factor* VIII *concentrate in various dilutions is demonstrated.*

4. *Fibrinogen and fibrin derivatives*

It has been demonstrated clearly that malignancy is a disease entity in which the levels of soluble fibrin complexes and fibrinogen/fibrin degradation products are elevated in an extremely large number of cases. They can be detected by means of laboratory tests even in patients not showing any clinical evidence of hemorrhagic or thromboembolic diathesis (Fig. 3). In a study of 61 patients with various malignancies the value of the serum fibrinolytic split products were increased in 82% and the fibrin monomer tests were positive in 55% of the cases [20]. In the light of these data and the confirming results of other investigators [72] the determination of fibrinogen and fibrin derivatives would seem to be an important diagnostic method for clinical practice.

nogen as the underlying disease progressed. The main findings of these results were confirmed by the studies of other investigators [27, 74–76].

IV. PATHOGENESIS

The tendency for thromboembolic complications to develop can be correlated in patients with malignant diseases with various non-specific factors such as age, sex, cachexia, physical immobilisation, exsiccosis, cardiac insufficiency, treatment with corticosteroids and diuretics. The decrease in megacaryocytopoiesis in the bone marrow arising in malignant blood diseases or as a result of aggressive chemotherapy with the ensuming thrombocytopenia would alone offer a sufficient explanation of a serious bleeding tendency. A depression of fibrinogen

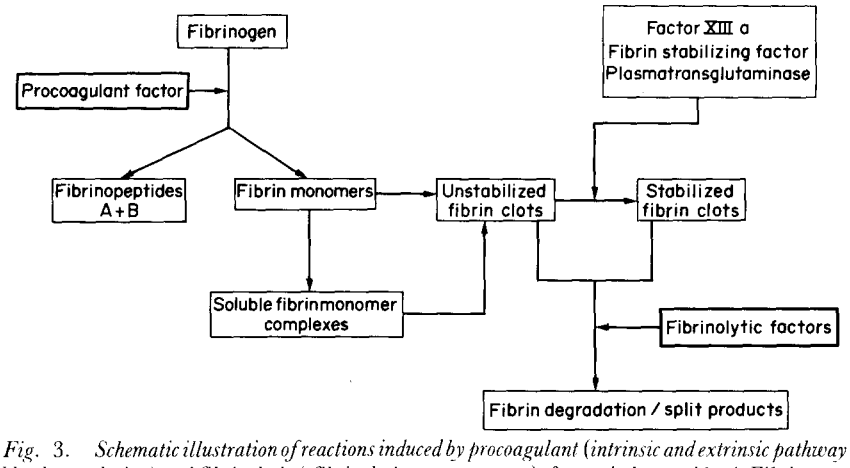

Fig. 3. Schematic illustration of reactions induced by procoagulant (intrinsic and extrinsic pathway of blood coagulation) and fibrinolytic (fibrinolytic enzyme system) factors in human blood. Fibrinogen as well as intermediates of fibrin formation (fibrin monomers and soluble fibrin monomer complexes) and fibrin degradation/split products are often elevated in patients with malignant diseases and can be estimated by simple laboratory tests.

5. *Platelet and clotting factor survival studies*

According to the studies of Slichter and Harker [73] the survival of labelled platelets and fibrinogen decrease parallel to one another in cancer patients. Grouping the data according to disease categories revealed a progressively shorter survival of the labelled components in chronic leukemias, metastatic solid tumors, lymphomas and sarcomas, carcinoma of the prostate and acute leukemias. In those patients, who died within 2 weeks of the study, platelet and fibrinogen survivals averaged 1 day, regardless of their disease classification, compared with the normal values of 9.5 ± 0.6 and 5.1 ± 0.3 days, respectively. In 5 out of 8 patients for whom there was objective evidence of response to chemotherapy or radiotherapy, there was a significantly prolonged survival of platelets and fibrinogen towards normal values. In contrast 5 patients with unresponsive disease showed an increased consumption of platelets and fibrinogen as the underlying disease progressed.

synthesis in the liver can be expected in the course of L-asparaginase therapy [77–79].

1. *Intravascular coagulation and fibrinolysis (ICF) syndromes*

Malignant diseases tend to be associated with a hypercoagulable state ("chronic disseminated intravascular coagulation") together with clinical and subclinical abnormalities of the hemostatic system, e.g. thrombocytosis, elevated levels of fibrinogen and other clotting factors, shortening of platelet and fibrinogen survival, elevated levels of fibrin monomers and soluble fibrin monomer complexes. At the same time fibrinogen/fibrin split products are elevated too, indicating a low grade activation of the fibrinolytic enzyme system. Wide-spread intravascular fibrin deposits are not detectable at autopsy. The term "intravascular coagulation with fibrinolysis (ICF)" was introduced to stress the close relationship between accelerated intravascular

coagulation and activated fibrinolytic mechanisms [80]. Diagnostically the most confusing cases of the ICF-syndrome are those with milder abnormalities and little or no bleeding. In many cases, however, the diagnosis can be established by the combination of characteristic laboratory findings.

Malignant tumors are thought to release procoagulant and fibrinolytic factors which are assumed to be the trigger mechanism responsible for the enhancement of the pathological process (Fig. 3). Procoagulant and fibrinolytic materials have been demonstrated in solid tumors as well as in leukemic cells [81–89]. At the present it must remain undecided however, whether this is the only trigger mechanism or whether others, which are primarily independent from clotting mechanisms, are not of importance as well. A possible limited or enhanced clearance function of the reticuloendothelial system for intermediates of the clotting process has to be taken into account. Interestingly enough, in some patients with acute leukemia an increased fibrinogen catabolism has been demonstrated without there being any clinical or laboratory evidence of intravascular coagulation [74].

2. *Disseminated intravascular coagulation and consumption coagulopathy*

The state of blood hypercoagulability and the intravascular coagulation–fibrinolysis syndrome in malignant diseases can develop to such a point that it gives rise to disseminated intravascular coagulation and consumption coagulopathy. Particularly characteristic for such a state are thrombotic and bleeding manifestations and thrombocytopenia as well as the reduced activity of most clotting factors. In most patients respiratory and renal insufficiency as well as symptoms of prolonged shock are apparent due to fibrin deposits in the microcirculation. This dangerous complication is not frequent, however, it is a constant finding in acute promyelocytic leukemia [8, 9, 90–95]. In other forms of acute leukemia the syndrome is rarer. There are only casuistic reports of acute disseminated intravascular coagulation and consumption coagulopathy in solid tumors describing in particular carcinomas of the prostate and pancreas. It is not fully understood, why some patients with malignant diseases develop the severe complication of acute disseminated intravascular coagulation and consumption coagulopathy while others do not [96]. It has been suggested that besides febrile episodes and endotoxaenemia particularly cytostatic chemotherapy could be an important accelerating factor in leukemias [97–99]. This seems not to be true, however, for patients with solid tumors [100].

3. *Microangiopathic hemolytic anemia*

The syndrome is characterized by elevated levels of plasma hemoglobin and erythrocyte fragments (schistocytes) in the peripheral blood smears. It is associated with thrombocytopenia and other irregularities in the blood clotting mechanism. Microangiopathic hemolytic anaemia is obviously a rare complication in metastasing carcinomas [101–103]. Carcinomas of the breast and stomach were the most frequent primary tumors, but single cases of metastatic carcinomas of colon, gall-bladder, pancreas, lung and prostate as well as malignant hemangioendotheliomas, have been known to be complicated by a microangiopathic hemolytic anemia.

Experimental data suggest that schistocytosis and hemolysis in microangiopathic hemolytic anemia is the result of mechanical erythrocyte fragmentation in diseased small blood vessels [104]. Pathological changes of small blood vessels in metastatic carcinoma due to intravascular tumor cell aggregates and fibrin deposits have been described [105].

Normally, microangiopathic hemolytic anemia is a late and fatal complication of metastatic carcinoma. However, quite often this type of anemia is inherent in the underlying tumor.

V. DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS

Venous thromboembolism, lung embolism and the appearance of bleeding tendency may be the first clinical symptom indicating the presence of a malignancy in patients not suffering from other predisposing diseases. The following tests should be included in a laboratory program with hemostatic parameters for early cancer diagnosis: evaluation of schistocytosis in peripheral blood smears, platelet counts, fibrinogen level, thrombin time, thromboplastin time and partial thromboplastin time, simple tests for detection and estimation of fibrin monomers and soluble fibrin monomer complexes (ethanol gelation test: Godal and Abildgaard [106]; protamine sulphate test: Lipinski and Worowski [107]; Niewiarowski and Gurewich [108]) as well as fibrin degradation products (hemagglutination inhibition test: Merskey *et al.* [109]). It should be pointed out that none of these tests giving pathological results in cancer patients is pathognomonic of such a disease as they may be seen also in non-malignant diseases. However,

they can be used as an additional diagnostic information. It has been clearly demonstrated that the regular control of hemostatic laboratory tests is helpful in estimating the course and prognosis of malignant diseases. It is important to be able to predict fatal complications such as acute consumption coagulopathy or microangiopathic hemolytic anemia. Laboratory evidence of intravascular coagulation and fibrinolysis syndromes disappears, when tumor regression is achieved.

Cancer patients with thromboembolic complications are treated according to the common rules of internal medicine. In patients with thrombocytopenia alone due to bone marrow insufficiency platelet transfusions will be able to get a bleeding tendency under control.

The therapy of intravascular coagulation and fibrinolysis syndromes should always be directed towards the primary disease, and measures should be taken to correct anything that exaggerates the coagulopathy, such as sepsis, shock or acidosis. Experience clearly indicates that no specific therapy other than supportive measures need be undertaken when the intravascular clotting is so minor and short lived that it does not contribute to either morbidity or mortality. However, the administration of heparin should be considered in cases where a thrombotic or bleeding tendency presents an immediate threat to the patient's life and where it is not possible to reverse the underlying mechanism triggering the intravascular clotting. The use of other agents such as blood transfusions, cryoprecipitates, platelet concentrates and antifibrinolytic drugs must be decided anew in each individual case [110]. The use of oral anticoagulants or antiplatelet agents (acetylsalicylic acid, dipyridamol) has not yet proved successful with these indications.

The adequate treatment of acute disseminated intravascular coagulation with consumption coagulopathy presents big therapeutic problems. Some authors have suggested the use of fibrinolytic agents such as streptokinase or urokinase, but as yet not much experience has been acquired in the field. The experiences with heparin therapy are conflicting [96, 111]. They include success with short term heparin therapy combined with specific treatment of the underlying disease, favorable laboratory response but clinical failure as well as both clinical and laboratory failure. It should be given, however, in cases of acute promyelocytic leukemia [112] and should be combined with fibrinogen, platelet concentrates and plasma fractions.

Heparin therapy is not appropriate for cancer patients with microangiopathic hemolytic anemia, unless there is laboratory evidence of disseminated intravascular coagulation and bleeding tendency. When trying to gain control over hemolysis and thrombocytopenia, only chemotherapy supported by platelet and erythrocyte transfusions seems to be beneficial. The use of antiplatelet drugs is still under discussion.

VI. CONCLUSIONS

From the clinical and laboratory data summarized in this review it becomes evident that the evaluation of the hemostatic system in patients with malignant diseases is important. A hypercoagulable state of the blood is a constant finding and can be diagnosed by laboratory procedures. Malignancy is associated with an increased consumption of platelets and fibrinogen and the turnover rate of these factors is related to the type and extent of the disease. From a pathogenetic point of view this seems to be due to an intravascular coagulation and fibrinolysis syndrome. Acute disseminated intravascular coagulation and consumption coagulopathy is a common complication in acute promyelocytic leukemia, but it is rare in other forms of malignancies. A hemostatic disorder is most effectively treated with antitumor therapy in order to reduce or remove the underlying lesion. Antithrombotic drugs such as heparin may prevent thromboembolic disorders. In the actively bleeding patient, transfusion therapy presents a better method of getting the bleeding under control in the first instance, than a manipulation of the hemostatic system. In general, it can be said that the pathogenesis, diagnosis, prevention and treatment of hemostatic abnormalities in cancer patients certainly deserves further clinical and basic studies. The laboratory techniques used to identify a hemorrhagic or thrombotic tendency as well as the therapeutic regimens used to correct such a tendency are not effective in all patients. Investigations are required to understand the mechanism and significance of impaired platelet function in many cases of malignant diseases. The reason for isolated coagulation factor deficiencies in acute leukemia (factors I, V, X, XIII) is not yet fully understood. The question must be clarified as to whether a reduced or enhanced clearance function of the reticuloendothelial system contributes to the coagulation disorders in malignancy by more or less eliminating the precursors and intermediates of the coagulation and fibrinolytic enzyme system. From a clinical point of view it would be important to know whether there is a correlation

between elevated levels of soluble fibrin monomer complexes in blood and the clinical incidence of thrombosis.

In the present paper, experimental results concerning the local involvement of hemostatic mechanisms in the formation of metastasis were not considered. Preliminary studies in man using anticoagulant drugs in cancer chemotherapy were excluded also. Both topics have been discussed in detail elsewhere [113–124].

Furthermore, recent animal model systems for studying the mechanisms of hemorrhagic and thrombotic tendencies in malignant diseases [125–126] were not reviewed.

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Plasma Levels and Biotransformation of Infused Daunorubicin and Daunorubicin–DNA Complex in Rabbits: A Preliminary Report

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Abstract—*Plasma levels and biotransformation of daunorubicin and daunorubicin–DNA complex were studied in rabbits after an i.v. bolus injection and during and after a* 4 hr *infusion period. The administered dose was* 5 mg/kg *expressed as the free drug.*

The levels of daunorubicin (DNR) and its active metabolite, daunorubicinol (DNR-ol), were determined on plasma extracts by high-pressure liquid chromatography.

The results demonstrated a slower disposition of the complex. In addition, a five-fold higher DNR-ol plasma level was achieved at the end of the infusion of the complex as compared with the free DNR infusion.

Incubations of DNR and DNR–DNA complex with whole rabbit blood indicated also a slower disposition of the complex.

Cell DNR-reductase activity in the rabbit was intense. Indeed, DNR-ol appeared rapidly during infusion of free drug and complex and during in vitro *incubation with whole rabbit blood.*

INTRODUCTION

DAUNORUBICIN (DNR), an anthracyclinic cytostatic antibiotic produced by *Streptomyces peucetius*, is very active in the treatment of several hematologic malignancies.

A lysosomotropic complex of DNR with DNA, extracted from calf thymus (Sigma, type V) or from herring sperm (Sigma, type VIII), has been prepared by Trouet. The complex injected into leukaemic mice was more active than the free drug [1].

The disposition of the complex in mice has recently been described. However, the authors measured the total plasma levels by fluorescence and did not attempt to separate the parent drug from its active metabolite, daunorubicinol (DNR-ol) [2].

Recently we described a fast, easy, and sensitive, method for the determination of DNR and DNR-ol in plasma by high-pressure liquid chromatography [3].

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The aim of this work was to estimate the plasma levels of DNR and its metabolite, DNR-ol, in normal rabbits during and after a 5 mg/kg i.v. infusion of either DNR as free drug or complexed with DNA.

A four-hour infusion period was preferred, since fast i.v. injection of the same dose of the complex required a too large volume (12.5 ml/kg), which resulted in an acute heart failure.

Studies on the *in vitro* metabolism of DNR and its complex in the whole rabbit blood were also performed.

MATERIAL AND METHODS

Normal F_1 rabbits, males and females, weighing about 3 kg, were used. No sex linked difference was observed for the measured parameters.

Pure reference samples of DNR and DNR-ol were obtained as the hydrochlorides from Rhône-Poulenc (France). Commercial daunorubicin-HCl (Cerubidine, Spécia, France) was administered to the rabbits.

For the preparation of the complex, a solution of DNA (4.68 mg/ml in NaCl 0.9%) from

herring sperm was used.* The solution was autoclaved for 15 min at 120°C and slowly cooled down. After cooling, appropriate amounts of DNR were added in order to obtain a molar ratio of 20 [1].

In vivo *studies*

In some experiments, uncomplexed DNR was injected i.v. (5 mg/kg in NaCl 0.9%) as a bolus. Intravenous fast injection (4 min) of the DNR–DNA complex was performed in 5 animals.

Slow i.v. infusions, at a constant rate of 12 ml/hr, were given by means of a Braun-Unita-I infusion pump. The 5 mg/kg DNR and DNR–DNA complex solutions were adjusted to a final volume of 48 ml by addition of NaCl 0.9%. Injections and infusions were made into the lateral vein of the ear.

Blood samples (5 ml) were collected, from a polyethylene catheter inserted in the carotid artery, in tubes containing EDTA di-K as anticoagulant and immediately centrifuged at 0°C. The plasma was removed and stored at −20°C until analyzed.

Electrocardiogram, blood pressure and rectal temperature of the treated rabbits, were monitored during the whole infusion period (Hewlett-Packard 7754 A-7803 B).

Chromatographic analysis

The extraction procedure and the high-pressure liquid chromatography have previously been reported [3].

One millilitre plasma samples containing adriamycin as internal standard were extracted with a mixture of methylenechloride and isopropanol (92:8 v/v).

The extracts were injected on a 5 μm silica microsphere column, eluted at 4500 psi (flow-rate 0.9 ml/min) with a quaternary solvent mixture (methylenechloride/methanol/25% ammonia/water 90:9:0.1:0.8 v/v). The absorbance of the eluates was monitored at 490 nm (Du Pont 848–837). Results were calculated from a calibration curve. Each extraction and each determination were done in duplicate.

With this method, plasma levels as low as 10 ng/ml of DNR or DNR-ol could be detected. The recovery from rabbit plasma was 90 ± 3% for both compounds.

For some controls, thin-layer chromatography on silica gel plates was performed, according to Bachur [4].

In vitro *studies*

Fifty millilitres of fresh heparinized whole rabbit blood were incubated with 100 μg DNR or DNR–DNA complex in a shaking and heating water bath (Lab Line Instruments, model 3564) under O_2/CO_2 (93.5/6.5 v/v) at 39 ± 0.5°C.

Five millilitre aliquots were drawn at various times (1, 5, 15, 30, 60 and 120 min), and DNR and its metabolite levels determined as described above.

The Student's *t*-test for independent means was utilized for all statistical analyses.

RESULTS

Infusion of pure DNA solution, free DNR or DNR–DNA complex, did not induce any significant change in the electrocardiogram, the blood pressure or the rectal temperature.

In contrast, fast i.v. injection of the DNR–DNA complex (about 40 ml in 4 min) induced acute cardiac failure in 2 of 5 rabbits. A more concentrate DNA solution could not be injected because of its high viscosity. Moreover, a dosage reduction would not allow any comparison with the other experimental data.

Plasma levels

(a) Intravenous bolus injection of 5 mg/kg free DNR in 4 rabbits (Fig. 1).

A high DNR-ol plasma level was achieved at the fifth minute. Thereafter, plasma levels of the parent drug and its metabolite declined rapidly. The plasma levels of the metabolite remained always higher than the levels of the parent drug. The clearance curve could apparently be fitted to the sum of three exponential terms. The half-life of DNR at the elimination phase (γ-phase) was about 300 min. Because of the lack of enough time points during the α and β phases, half-lives could not be calculated for these phases.

(b) Fast i.v. injection of DNR–DNA complex (5 mg/kg DNR) in 5 rabbits.

Because the severe side effects (acute heart failure and premature death) induced by the fast injection, the dispersion of the data was too large and did not allow evaluation of the results in this group.

(c) Free DNR 4 hr infusion (5 mg/kg) in 4 rabbits (Fig. 2).

During infusion, the DNR-ol plasma levels increased steadily, exceeding the DNR plasma level after 2 hr. The plasma decay of DNR was fast after discontinuation of the infusion. The decay of DNR-ol was slower.

(d) DNR–DNA complex 4 hr infusion (5 mg/kg DNR) in 7 rabbits (Fig. 3).

A progressive increase in the plasma levels of the 2 compounds was obtained; the DNR-ol concentration reached the level of 2/3 of the

*Kindly provided by Trouet.

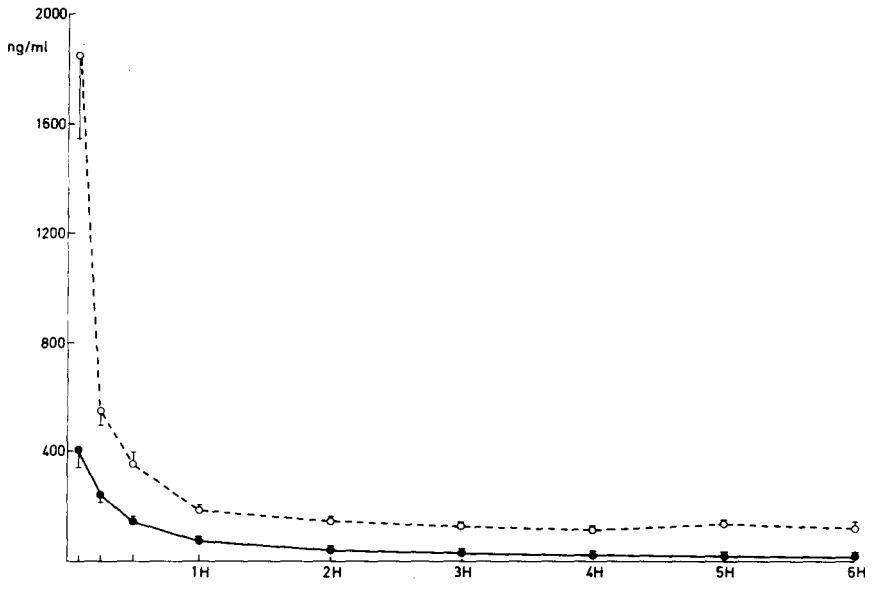

Fig. 1. Daunorubicin (●) and daunorubicinol (○) plasma levels (ng/ml) ± *S.E.M. in 4 rabbits after i.v. injection of* 5 mg/kg *daunorubicin as a bolus.*

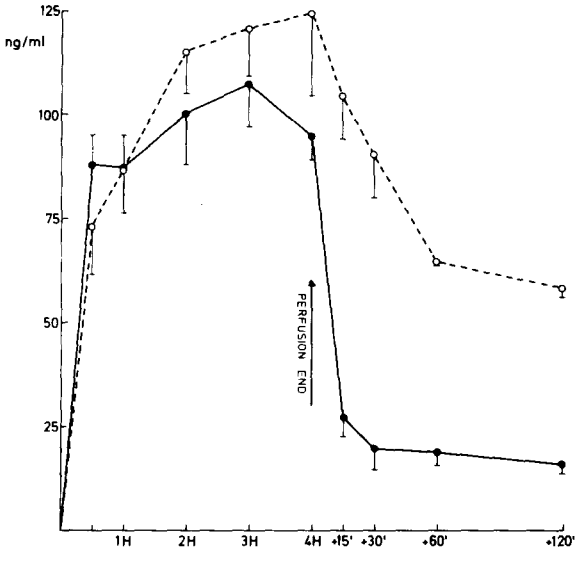

Fig. 2. Daunorubicin (●) and daunorubicinol (○) plasma levels (ng/ml) ± *S.E.M. in 4 rabbits infused during* 4 hr *with* 5 mg/kg *of daunorubicin.*

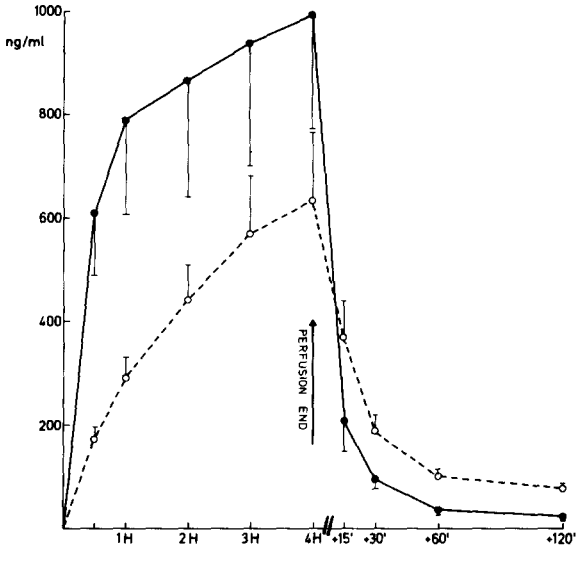

Fig. 3. Daunorubicin (●) and daunorubicinol (○) plasma levels (ng/ml) ± *S.E.M. in 7 rabbits infused during* 4 hr *with* 5 mg/kg *of daunorubicin–DNA complex.*

DNR concentration at the end of the infusion. Thereafter, the plasma decay of DNR and DNR-ol was similar to that observed after uncomplexed DNR infusion.

Statistical analysis of the data obtained during infusion of free DNR and DNR–DNA complex revealed a significant increase ($P \leqq 0.05$) at each time point for both DNR and DNR-ol plasma levels during infusion of the complex. After the end of the infusion, a significant increase in DNR levels was only observed at 15 and 30 min, while statistical significance was lost for the DNR-ol levels.

In vitro *studies*

Incubation of free DNR and DNR–DNA complex with whole rabbit blood (3 experiments, Table 1).

A fast decay of DNR from the plasma was observed during the incubations. However, the initial levels were higher with the complex. DNR-ol appeared rapidly in both cases. No significant difference could be observed between the DNR-ol levels, except at the last time point. The decay of both DNR and the complex was bi-exponential. The half-lives were 6 min 30 (α-phase) and 160 min (β-phase) for the free drug, 10 min (α-phase) and 70 min (β-phase) for the complex.

No further metabolite than DNR-ol was detected as controlled by thin-layer chromatography [4].

Table 1. *Daunorubicin and daunorubicinol plasma levels observed during* in vitro *incubation of whole rabbit blood with* 2 μg/ml *free DNR or DNR–DNA complex* (3 *experiments*)

	Free DNR incubation		DNR–DNA complex incubation	
Time (min)	DNR ng/ml ± S.E.M.	DNR-ol ng/ml ± S.E.M.	DNR ng/ml ± S.E.M.	DNR-ol ng/ml ± S.E.M.
1*	895	111	1807	79
5	411 ± 135	306 ± 121	924 ± 288	260 ± 118
15	216 ± 36	812 ± 223	493 ± 151	865 ± 273
30	51 ± 10	1085 ± 234	241 ± 66	1144 ± 164
60	17 ± 4	1276 ± 220	79 ± 25	1460 ± 280
120	13 ± 2	1480 ± 183	44 ± 12	1994 ± 99

*One experiment.

DISCUSSION

The plasma levels obtained after bolus injection of DNR are consistent with those previously reported [4]. However, a high plasma level of the metabolite DNR-ol was observed during the initial period (5 and 15 min). No explanation for this phenomenon can be put forward from our present data.

The plasma levels of both DNR and DNR-ol were significantly higher during infusion of the complex than during infusion of the free drug. This indicates that the disposition of the complex is slower and probably occurs by endocytosis [1]. If the disposition of the complex is indeed due to endocytosis, this process must be very active in the rabbit, since the plasma decay of the complex was very fast upon termination of the infusion: only the DNR plasma levels obtained at 15 or 30 min after infusion were significantly increased as compared to the free DNR infusion experiments.

The higher DNR-ol plasma levels observed during infusion of the complex are probably due to the binding of this compound by circulating DNA, since the binding sites of the DNR–DNA complex used in our experiments are not completely saturated [5].

Further experiments are needed to examine a possible slower biliary or urinary elimination of the complexed drug and to calculate the distribution volumes.

During the incubation of DNR and DNR–DNA complex with the whole rabbit blood, the half-lives of DNR plasma levels were different. In the case of the complex the α phase was slower whereas the β phase was markedly faster. In addition, the level calculated for the zero point was definitely lower in the free DNR incubation. This might indicate a stronger binding of the free drug to the blood cell membranes.

The plasma levels of the metabolite were similar during the whole incubation period, except at the last time point. As in humans [6], blood cell DNR reductase activity of the rabbit is probably highest in leukocytes. If the complex is taken up by endocytosis in these cells, this might explain its slower disposition during the initial phase as well as the similar plasma levels of DNR-ol found with the free drug and the complex.

In conclusion, the aim of this work was to compare plasma levels and biotransformation of DNR during a 4 hr infusion of DNR–DNA complex and of free DNR. It was found that the disposition of the complex was slower and that a five-fold higher DNR-ol plasma concentration was obtained with the complex. The slower disposition of DNR and the higher plasma levels of its active metabolite, DNR-ol, during infusion of the complex might explain the experimentally more sustained cytostatic activity of the complex.

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Studies of the Mechanism of Carcass Fat Depletion in Experimental Cancer

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Abstract—*Loss of body fat is commonly associated with both experimental and human cancers. A direct effect of cancer upon free fatty acid mobilization from host adipose tissue could potentially explain this lipid depletion. Rates of free fatty acid (FFA) production were measured in epididymal adipose tissue removed from normal-control rats and rats bearing the Walker* 256 *carcinoma either intramuscularly (i.m.) or intraperitoneally (i.p.). By the* 4th *day after i.p. transplantation and by the time the i.m. tumor mass reached* 4% *of body weight (b.w.), basal lipolytic rates were* 2–3 *times higher in adipose tissue from tumor bearing than age matched control rats. Body neutral lipids were not yet significantly reduced at this early time. The high rates of adipose tissue lipolysis were maintained as the cancer progressed and body neutral lipids became significantly reduced once the i.m. tumor attained* 6–7% *of b.w.*

The mechanism by which cancer causes this increased fatty acid mobilization is yet unclear. The Walker 256 *tumor might produce a lipolytic factor which directly stimulates adipose tissue lipolysis, since ascites serum from i.p. tumor bearing rats could stimulate adipose tissue lipolysis* in vitro. *However, blood serum from tumor bearing rats did not seem to stimulate adipose tissue lipolysis* in vitro *more than blood serum from control rats. Severe chronic stress seems an unlikely explanation for the early stimulated lipolysis in cancer, particularly the i.p. tumor. However, rats with even small i.m. tumors, although appearing stress free, possessed enlarged adrenal glands and somewhat atrophied thymus glands, classic markers for the presence of a stress situation. The cancer, in some unknown manner, might induce some special stress reaction.*

INTRODUCTION

Body lipids become progressively depleted in rats with the Walker 256 carcinoma [1–6], in mice bearing the Krebs-2 tumor [7] and in patients with various neoplasms [8, 9]. This depletion could be related to the growth of the tumor and could contribute to the debilitation of the host. Hyperlipidemia, consisting mainly of elevation of triglyceride-rich very low density lipo-proteins (VLDL), also occurs in experimental animals [3, 4, 10–14] and man [15, 16] with cancer. The decrease in body fat and increase in plasma lipid concentration could be secondary to increased mobilization of free fatty acids (FFA) from adipose tissue and subsequent conversion of these FFA to VLDL-triglyceride by the liver. VLDL-triglyceride derived from the host plasma could be an important source of the fatty acid for tumors. Brenneman *et al.* [12] recently reported that the ascites plasma formed with the Ehrlich ascites tumor contains large amounts of triglyceride-rich VLDL which are probably derived from the host's blood plasma and the Ehrlich cells can readily incorporate this triglyceride fatty acid [17]. Brenneman *et al.* suggest that "triglyceride-rich VLDL may serve as a transport vehicle for delivery of free fatty acids to the tumor" [12].

The mechanism by which the cancer leads to depletion of the host's body fat and to hyperlipidemia is unknown. It could be due to a cancer mediated stimulation of FFA release from host adipose tissue; however, this possibility has not been directly investigated. Others have speculated that the cancer might cause the host to produce lipolytic hormones [18, 19] and that the cancer itself might secrete lipolytic factors [7, 20, 21]. The present study examines the relationship between cancer and depletion of host body lipids by directly measuring *in vitro* production of FFA by epididymal adipose tissue from normal-control rats and from rats bearing the Walker 256 carcinoma, either intraperitoneally (i.p.) or intramuscularly (i.m.), at various stages of tumor growth. In addition, the ability of blood serum from normal and tumor bearing rats and of cell-free ascites serum

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*Reprint requests respondent.

to stimulate adipose tissue lipolysis was measured.

MATERIAL AND METHODS

Tumor maintenance

The Walker 256 carcinoma, obtained from the Arthur D. Little Co., Inc., Cambridge, MA, was maintained by weekly passage of 0.5–2×10^5 cells into the peritoneal cavity of male Wistar rats (50–100 g, Hilltop Lab Animals, Inc., Scottdale, PA) fed Purina rat chow. Groups of age and weight matched male rats were equally divided into control and test groups. Rats inoculated intraperitoneally (i.p.) with $1–2 \times 10^5$ cells died 7–8 days later and then possessed a tumor load of more than 10^9 cells. The tumor load on day 6 was about 0.5×10^9 ascites cells. Intramuscular (i.m.) tumors were maintained by injecting $0.5–5 \times 10^6$ ascites cells into the right thigh. These animals survived 4–5 weeks and possessed a tumor mass often exceeding 20% of total body weight.

Measurement of body neutral lipids

Quantitation of total body neutral lipids (mainly triglycerides) involved sacrificing age matched control and tumor bearing rats by decapitation, skinning the rats, mincing the hide, and putting the combined carcass plus minced hide through a meat grinder. The ground rat was quantitatively recovered by washing with one percent NaCl containing 2mM EDTA (Sigma Chem. Co., St. Louis, MO.), transferred to a large beaker and thoroughly homogenized in a polytron (Kinematica GMBH, Luzern, Switzerland) at low speed. Tumors were removed from the cancer bearing rats prior to grinding and homogenization. The homogenized rat mixture was transferred to a 1 liter volumetric, brought to volume with the NaCl solution, and a 10 ml aliquot was extracted into 200 ml of 2:1 chloroform:methanol (Spectroanalyzed, Fisher Scientific Co., Fair Lawn, NJ). The lipids were recovered essentially as described before [22]. Phospholipids were removed with silicic acid and the recovered neutral lipids were measured gravimetrically. Serum triglycerides were measured by the method of Lofland [23] and plasma FFA were quantitated as described below.

Measurement of adipose tissue lipolysis

Epididymal adipose tissue, immediately removed from decapitated rats, was incubated for 1 hr at 37°C in Krebs phosphate buffer (pH 7.4, no calcium, 2.5% fatty acid poor bovine serum albumin). Incubated systems contained 50–70 mg of minced adipose tissue pooled from 2 or more rats/ml. Samples of the incubation medium were taken for measurement of FFA by automated titration [24] after 6 min of equilibration at 37°C and after 60 additional min of incubation. Net lipolytic rate was expressed as μEq of FFA produced/g tissue (wet wt)/hr.

The effects of adding to the buffer either norepinephrine (Winthrop Labs, NY, NY), prostaglandin E_1 (Upjohn Co., Kalamazoo, MI), propranolol (Sigma Chem. Co., St. Louis, MO.), ascites serum or blood serum upon adipose tissue lipolysis were measured. Ascites serum is the cell-free fluid which accompanies the ascites cells isolated from the peritoneal cavity. Significant volumes of ascites serum (>2 ml) were collectable usually only after 6 days post-transplantation.

Measurement of triglyceride lipase activity

Lipase activity of ascites and blood serum was measured by a modification of the method of Guder *et al.* [5]. One ml of fresh serum was incubated for 1 hr at 37°C with 0.5 ml of buffer [pH 5–7, sodium cacodylate (0.1 M); pH 7–9.5, Tris-HCl (0.1 M), Fisher Scientific Co., Fair Lawn, NJ] and with either 0.5 ml of a 40% acqueous emulsion of purified triolein (Nutritional Biochem. Co., Cleveland, Ohio) or 0.5 ml of isotonic saline. Net lipolysis of triolein was calculated by subtracting the endogenous FFA production measured at each pH and expressed as μEq of FFA produced/ml of serum or liter of buffered-medium/hr.

Measurement of ascites or blood serum lipolytic activity

When blood or ascites serum was added to adipose tissue incubated *in vitro*, FFA production was increased. Since ascites serum was subsequently shown to contain lipase activity (Table 5 and Fig. 3), some or all of the apparent stimulation of lipolysis could be due to lipase attack of triglyceride normally present in ascites serum or to attack of triglyceride droplets dislodged from the adipose tissue pieces during incubation. Ascites serum from the Walker tumor contains about 70 mg % triglyceride [26] that is assumed present as VLDL-triglyceride [12]. In order to separate increased FFA production due to lipase activities from that due to stimulation of adipose tissue lipolysis, epididymal adipose tissue pieces, pooled from several normal rats, were incubated in either the plain Krebs phosphate buffer described above, or in this buffer containing ascites or blood serum at 33% or less by volume; respectively, called systems A and B (see Table 6). Adipose tissue-free medium recovered after incubation of the adipose tissue in buffer alone, system A, was centrifuged ($100{,}000 \times g$ for 45 min) to con-

centrate medium lipids, the bottom one-third volume or less was replaced with serum, and the mixture, now system C, was reincubated for 1 hr at 37°C. The concentration of triglyceride in system C (a tissue free incubation) is assumed to approximate the concentration present when adipose tissue and ascites serum were incubated together (system B). That is, system C is assumed to contain the same amount of medium lipids upon which ascites or blood serum lipases could act as was present in system B. Lipolytic activity of serum (the increased FFA production by adipose tissue due to stimulation by factors in serum) should therefore equal the total FFA produced when adipose tissue and ascites serum were incubated together (system B) *minus* the *sum* of the FFA produced as a consequence of basal lipolysis (estimated in system A) *plus* the FFA produced through the action of serum lipase (estimated in system C). That is, the lipolytic activity of serum should equal B − (A + C); see Table 6. This value might be somewhat high because of possible under estimation of the total ascites serum lipase activity. This might have occurred if ascites serum caused release from adipose tissue of lipase activating factors, tissue lipases, or triglyceride substrate. Such effects would have been missed with the current methods.

RESULTS

Effect of the Walker tumor on host total body neutral lipids and blood lipids

As compared with age and weight matched controls, rats bearing the Walker 256 tumor intramuscularly progressively lost body neutral lipids after the tumor reached about 6–7% of the total body weight (Table 1). When the tumor comprised 10% or more of the body weight, total neutral lipids fell to about one-half the level found in the age matched controls. Also, plasma FFA concentrations were increased in rats with large i.m. tumors (Table 2). In contrast, body lipids were not decreased in rats bearing the tumor intraperitoneally in ascites form (data not shown); although, plasma FFA levels were increased by day 4 of this tumor (Table 2). The ascites form of the tumor was almost always fatal at day 7 after transplantation; whereas, rats with the i.m. tumor survived 4–5 weeks. Circulating levels of triglycerides of control and tumor bearing rat groups ranged from means of about 80 to 120 mg/100 ml and were not statistically different.

Effect of the tumor on host adipose tissue lipolysis

Once the intramuscular tumor reached 4% of total body weight, basal lipolytic rates of epididymal adipose tissue removed from tumor bearing rats were 2–3 times higher than those from matched controls (Fig. 1). Also, as compared with controls, basal lipolytic rates were significantly elevated in rats with the i.p. tumor starting at day 4 after transplantation and continued elevated until day 7, the usual time of death (Fig. 1). Elevation of adipose tissue lipolysis for only 3–4 days prior to death is apparently insufficient time to result in the major loss of body fat in these i.p. tumor-bearing rats.

Table 1. *Total body neutral lipid concentration in rats bearing the Walker* 256 *carcinoma intramuscularly*

Tumor as (% Body wt) (b.w.)	Neutral lipid (% Total body weight)*	
	Tumor-bearing rats†‡	Tumor-bearing rats / Age matched control rats
1.70	5.713	1.089
3.34	5.731	1.049
5.60	4.956	0.985
6.51	3.928	0.824
7.61	4.962	0.708
9.90	4.298	0.501
10.62	3.422	0.611
11.91	1.843	0.526
12.29	2.656	0.474

*Rats, inoculated i.m. with 2×10^6 tumor cells and uninoculated controls, were sacrificed in pairs at intervals between 7 and 35 days after inoculation. The rats were homogenized and body lipids extracted and quantitated as described in "Material and Methods".

†Total neutral lipids of the whole body minus the tumor mass.

‡A plot of tumor weight (as % b.w.) vs total body neutral lipid concentration (as % b.w.) gave a straight line with a negative correlation coefficient of −0.90 that was significantly different from zero [$P(t) < 0.0025$].

Table 2. Plasma FFA concentrations in control and tumor-bearing rats

Rat type	No. rats	Day of tumor	Plasma FFA*† (μEq/l)
Group I			
Controls	6	—	255 ± 34
Tumor (i.p.)	6	4	436 ± 40‡
	7	5	370 ± 34‡
	10	6	453 ± 39‡
		Tumor as (% b.w.)	
Group II			
Controls	6	—	237 ± 27
Tumor (i.m.)	6	9.5 ± 1.1	519 ± 61‡

*Values are means ± one S.E.M.
†Age matched controls were sacrificed along with the tumor bearing rats.
‡$P(t)$ of diff. from the respective control group is < 0.01 (Student's t-test).

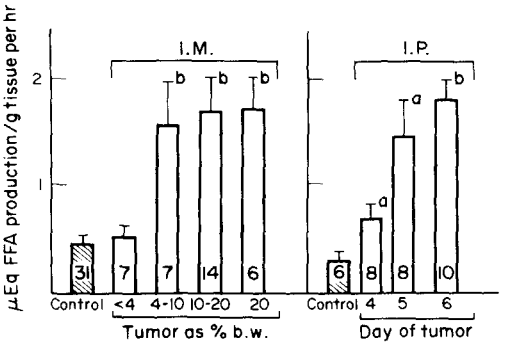

Fig. 1. In vitro basal lypolytic rates of epididymal adipose tissue from host rats bearing the Walker 256 carcinoma either intramuscularly (i.m.) or intraperitoneally (i.p.). Bars are the means ± S.E.M. of the net lipolytic rates determined for the underlined number of individual control rats, ▨, or tumor bearing rats, □. Epididymal fat pads from rats were minced and incubated for 1 hr at 37°C in Krebs phosphate buffer (pH 7.4, no calcium) containing 2.5% bovine-serum albumin (fatty acid poor). Incubation flasks contained 50–70 mg of tissue/ml. P(t) of difference from control activity are < 0.025 for a and < 0.001 for b.

Indices of stress in tumor-bearing rats: food consumption and weights of adrenals and thymus

Rats with the Walker 256 tumor growing intraperitoneally consumed the same amount of food as matched controls until the 24 hr immediately prior to death; i.e. day 6–7 (data not shown), and had adrenal and thymus gland weights similar to those of control animals (Table 3). Also, rats with large i.m. tumors (about 17% of body weight) demonstrated no gross signs of anorexia; however, rats with even small i.m. tumors possessed significantly larger adrenal and smaller thymus gland weights than controls (Table 3). In stress adrenal weight can increase and thymus weight decrease [27].

Search for lipolytic factors in cell-free ascites serum

The possibility that the Walker 256 tumor secretes lipolytic factors was investigated initially by incubating epididymal adipose tissue from normal rats in buffer containing varying concentrations of fresh cell-free ascites serum obtained from rats bearing a 7 day i.p. tumor. Addition of ascites serum to the incubation medium resulted in a dose-related increase in FFA production (Table 4). Since we subsequently observed that ascites serum also contains appreciable lipase enzyme activity (Table 5), some or all of the apparent stimulation of lipolysis could be due to lipase hydrolysis of lipids normally present in ascites

Table 3. Adrenal and thymus weights in control and tumor-bearing rats

Rat type	n Rats	Day of tumor or % tumor (of b.w.)	Adrenal wt* (mg/100 g b.w.)	Thymus wt* (mg/100 g b.w.)
Control	21	—	17.5 ± 0.6	313 ± 13
Tumor (i.p.)	8	Day 6	18.4 ± 0.8	347 ± 6†
Tumor (i.m.)	8	2.6 ± 0.6	19.1 ± 1.0‡	274 ± 13§
	4	8.9 ± 0.5	19.0 ± 1.0	244 ± 12§
	6	15.3 ± 1.2	20.8 ± 0.6§	243 ± 19§

*Values are mean organ wet weight ± S.E.M.
†$P(t)$ of difference from control < 0.01.
‡$P(t) < 0.005$.
§$P(t) < 0.001$.

Table 4. Effect of cell-free ascites serum on basal lipolysis of epididymal fat from normal rats

% Ascites serum in medium (% v/v)*	μEq FFA produced/g tissue (wet wt)/hr†
0 (Control	0.78 ± 0.26
2	1.13 ± 0.11
10	1.61 ± 0.12‡
20	2.22 ± 0.45‡

*Ascites serum was recovered from the peritoneal cavity of rats with a 7 day tumor. Samples of adipose tissue from minced and pooled epididymal fat pads from several normal rats were incubated for 1 hr at 37°C in the Krebs phosphate buffer containing varying concentrations (by volume) of ascites serum. The incubated systems contained 50 mg of tissue/ml.

†Values are the mean ± S.E.M. of 3 experiments.

‡$P(t)$ of difference from control (0% ascites serum) < 0.05.

Table 5. Lipase activity of cell free ascites serum

Substrate	No. exp.	μEq FFA produced/L medium/hr*† pH 5	pH 7.5
Endogenous	5	20 ± 10	55 ± 21
Triolein	5	244 ± 25	433 ± 31

*Values are means ± S.E.M.

†One ml of fresh ascites serum was incubated for 1 hr at 37°C with 0.5 ml of 0.1 M sodium cacodylate buffer (pH 5 or 7.5) plus 0.5 ml of either isotonic saline (endogenous substrate) or of a 40% aqueous emulsion of purified triolein.

serum or to hydrolysis of lipid droplets released from the adipose tissue pieces during incubation. Lipase activities in ascites serum did indeed account for some of the increased FFA production seen upon incubation of adipose tissue with ascites serum; however, it is unlikely that it accounted for all of the increase (Table 6). The apparent lipolytic activity in ascites serum was unaffected by concentrations of prostaglandin E_1 and propranolol which clearly blocked the lipolytic response to norepinephrine (Fig. 2).

Comparison of lipolytic activity of blood serum from control and tumor-bearing rats

Blood serum from normal-control and tumor-bearing rats was compared for ability to stimu-

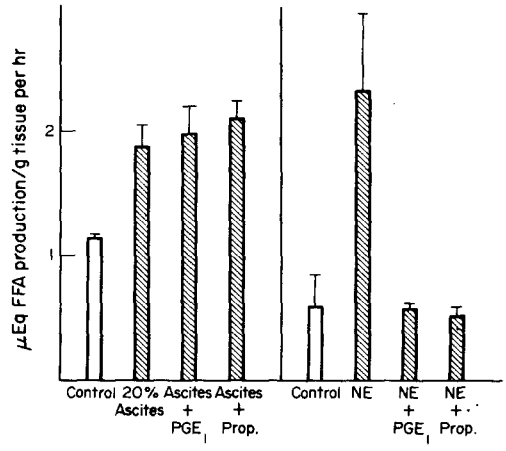

Fig. 2. Effects of prostaglandin E_1 (PGE_1) and propranolol (Prop) upon ascites serum and norepinephrine (NE) stimulated lipolysis. Samples of adipose tissue from minced and pooled epididymal fat pads of normal rats were incubated for 1 hr *at* 37°C *in buffer (same as in Fig.* 1*) containing no additions (Control) or containing various combinations of ascites serum* (20%, v/v), PGE_1 (1 μM), *Prop* (0.1 μM), *and NE* (1.6 μM). *Bars are the mean ± S.E.M. of* 3 *experiments.*

Table 6. Comparison of lipase versus lipolytic activity of ascites serum

System*	μEq FFA produced/ L incubated system/hr†
A. Adipose alone	26 ± 5 (0.35 ± 0.07)‡
B. Adipose + ascites serum (33% by vol)	149 ± 18
C. Cell-free medium from A + ascites serum (33%)	56 ± 10 = Lipase activity
D. Lipolysis stimulated by Ascites serum = B − (A + C)	67 ± 14 (1.74 ± 0.20)§

*Samples of adipose tissue pieces (0.6 g) from minced and pooled epididymal fat pads of several normal rats were incubated in 9 ml of the Krebs phosphate buffer (pH 7.4), System A, or in 6 ml of buffer plus 3 ml of cell-free ascites serum, System B. The buffered medium recovered after incubation of System A was reincubated with ascites serum, System C.

†Values are the mean ± S.E.M. of 6 experiments.

‡Lipolysis expressed as μEq FFA produced/g tissue (wet wt)/hr.

§$P(t)$ of difference between B (149 ± 18) and A + C (82 ± 7) is < 0.01.

late lipolysis of control rat adipose tissue. Since blood serum from both control and tumor rats contained significant lipase enzyme activity at pH 7.4 (Fig. 3), it was necessary to conduct the "balance type" experiment described before (see Methods) for ascites serum in order to separate blood serum lipolytic activity from lipase activity. Blood serum from normal rats stimulated lipolysis in a dose-related manner, producing a maximum 3–4 fold increase above basal levels (Table 7). Rather surprisingly, blood serum from rats with ascites or intramuscular tumors did not stimulate adipose tissue lipolysis more than that from control rats. Serum from the i.m. tumor bearing rats actually seemed to be less active.

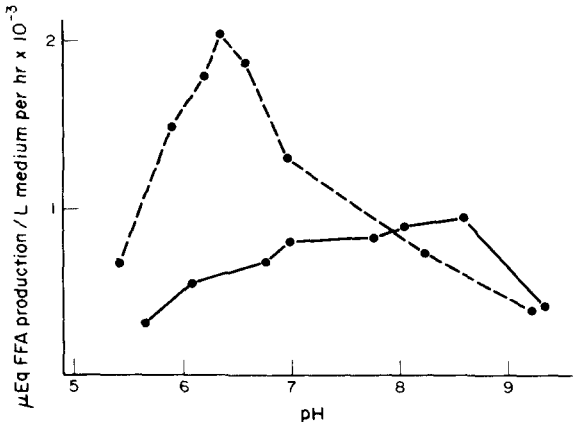

Fig. 3. Effect of pH on lipase activity of blood serum from control rats, ———, and of ascites (i.p.) tumor bearing rats, – – – – . The substrate was triolein. Lipase assays were conducted by incubating 1.0 ml *of blood serum for* 1 hr *at* 37°C *with* 0.5 ml *of buffer* [pH 5–7, *sodium cacodylate* (0.1 M); *pH* 7–9.5, *Tris HCl* (0.1 M)] *and* 0.5 ml *of either a* 40% (v/v) *aqueous emulsion of purified triolein or isotonic saline. Individual points are the mean of* 2 *experiments.*

DISCUSSION

The present study demonstrates that rats bearing the Walker 256 carcinoma either intraperitoneally or intramuscularly begin to mobilize adipose tissue FFA early in the development of the tumor. This increased mobilization precedes or parallels the depletion of carcass neutral lipid in rats with the i.m. tumor and is seen to occur when the tumor mass is still small and before the tumor is life-threatening. Body neutral lipids were not significantly decreased in rats with the i.p. tumor apparently because of the short period during which FFA mobilization was elevated. Increased adipose tissue lipolytic rates were first recognized on day 4 after i.p. transplantation of the tumor; however, the animals died on day 7–8. Many different mechanisms could theoretically explain this cancer-related early stimulation of fat mobilization.

The cancer could secrete factors which directly stimulate lipolysis or which cause the host to produce more circulating lipolytic hormones. Recently Farron and Lightholder [28] reported that the Walker 256 carcinoma cultured *in vivo* along with foetal liver explants produces a diffusable factor(s) which directly inhibits the accumulation of certain embryonic liver enzymes. In the present study, cell-free ascites serum recovered from rats bearing this tumor intraperitoneally did stimulate adipose tissue lipolysis. And, this apparent lipolytic factor(s) in ascites serum is probably not a catecholamine or a hormone which stimulates lipolysis through activation of adenylate cyclase, since neither propranolol nor prostaglandin E_1 antagonized the lipolytic activity of ascites serum at doses

Table 7. Stimulation of lipolysis by blood serum from control and tumor-bearing rats

Serum source	No. samples tested*	% Serum (v/v)	Stimulation of basal lipolysis (%)†‡
Control rats	2	6.6	39.1 (30.8 and 47.4)
	12	16.7	278 ± 53
	8	33.3	347 ± 46
I.P. Tumor rats (day 6)	2	6.6	71.4 (55.6 and 87.2)
	2	16.7	214 (259 and 168)
	9	33.3	400 ± 69
I.M. Tumor rats (tumor = 13.9 ± 2.2% b.w.)	12	16.7	120 ± 37§

*Serum samples from individual rats.

†The values are means and means ± S.E.M. Correction for serum lipase activity was done as described for the experiments reported in Table 6.

‡Adipose tissue basal lipolysis in the absence of added serum was an average 1.19 ± 0.05 μEq FFA/g/hr.

§$P(t)$ of difference from control serum at 16.7% serum is < 0.005.

which clearly block the lipolytic response to norepinephrine (Fig. 2). Propranolol specifically blocks the beta-receptor mediated catecholamine stimulation of adipose tissue lipolysis [29] and prostaglandin E_1 is a general antagonist of substances which increase lipolysis by activation of adenylate cyclase [30]. Whether this ascites factor enters the systemic circulation *in vivo* is unclear, since blood serum from both i.p. and i.m. tumor bearing rats failed to stimulate adipose tissue lipolysis *in vitro* more than that from normal rats. These findings could suggest that the Walker tumor neither secretes quantitatively significant amounts of lypolytic factors into the circulation nor causes the host to produce more circulating lipolytic hormones. However, the activity of certain lipolytic factors in blood serum could have been missed with the present methods. For example, labile substances with a short biological half-life could have been lost prior to start of the incubations. On the other hand, the activities of some lipolytic factors could have been overlooked because our incubations were too short. For example, the lipolytic activity of pituitary diabetogenic protein is independent of cAMP and requires more than 1 hr incubation with adipose tissue *in vitro* to be expressed [31–33]. Pituitary diabetogenic protein secretion is greatly increased in patients with lipoatrophic diabetes [34], a disease strikingly similar to advanced cancer in that both conditions can result in generalized subcutaneous lipoatrophy and hyperlipidemia.

The general mechanisms of relative caloric deficiency or stress would appear inadequate alone to explain the observed increased adipose tissue lipolysis and depletion of carcass neutral lipid in cancer. The loss of body lipids is reduced but not prevented in both animals [35] and humans [36] bearing cancer by increasing caloric intake through force feeding. In the present study anorexia was not evident in rats with i.p. tumors until just 24 hr prior to death and there was no great difference in food consumption between groups of normal rats and ones bearing large i.m. tumors. Baldwin *et al.* [37] also observed no decrease in food intake or weight gain of rats bearing large subcutaneous Walker 256 tumors (4–6 cm dia). Tumor growth is probably more dependent upon the host than host growth upon tumor activity. For example, glucose utilization *in vivo* by the Walker tumor is dependent upon the host's plasma glucose concentration [38]. Our finding of an increase in adrenal gland and decrease in thymus gland weights in rats with even small i.m. tumors indicates the presence of some stress at early stages of tumor growth. Whether this stress is resulting in the increased fatty acid mobilization, perhaps secondary to increasing the tone of sympathetic fibers innervating adipose tissue, must yet be determined.

In conclusion, the results of the present study indicate that the loss of body lipid in cancer results from a primary effect on mobilization of free fatty acid from the host's adipose tissue and that this increased mobilization begins early in the growth of the tumor. The mechanism by which the cancer causes this fatty acid mobilization is still unknown. It possibly could involve tumor production of a lipolytic factor which directly affects adipose tissue lipolysis and (or) induction, in some unknown manner, of a special stress reaction. Understanding how cancer leads to excessive lipid mobilization in the host opens the possibility of preventing this action and this could be potentially important for retarding tumor growth and improving the well-being of the patient.

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DNA Synthesis Inhibition by Dimethylnitrosamine in Regenerating Rat Liver*

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Abstract—*The DNA synthesis was measured in regenerating liver of rats injected with various doses of dimethylnitrosamine at different times after partial hepatectomy, and sacrificed from* 20 *to* 264 hr *after surgery* (1 hr *after* [^3H]-*thymidine injection*).

DMNA (9 mg/kg) *given* 30 min *after partial hepatectomy deeply inhibits the first two DNA synthesis waves, significant incorporation of* ^3H-*thymidine being observed only* 56 hr *after surgery.*

When administered 24 hr *after partial hepatectomy* (*at the peak of DNA synthesis in control animals*), *the same dose of DMNA quickly inhibits the* [^3H]-*thymidine incorporation, which only starts again with an overshoot above control values*, 96 hr *after surgery.*

Doses of 2, 4, 9 *and* 18 mg/kg*DMNA were given* 30 min *or* 24 hr *after partial hepatectomy, and the DNA specific activity was measured at* 28 hr *after surgery* (1 hr *after injection of* [^3H]-*thymidine*). *The DNA synthesis inhibition was usually proportional to the dose, but very similar when the drug was injected* 0.5 *or* 24 hr *after surgery. From this observation, it may be concluded that DNA synthesis inhibition by DMNA administered after partial hepatectomy originates more from template alteration than from delay in enzyme synthesis.*

INTRODUCTION

DIMETHYLNITROSAMINE (DMNA) is a powerful carcinogen inducing liver tumours in the rat if administered chronically over a long period of time [1], or as a single dose if given during liver regeneration following partial hepatectomy [2–5].

The most striking biochemical effect of this drug is nucleic acids alkylation, mainly in the liver, which is the site of the DMNA metabolism [1]. Quantitatively, the main alkylation site is the N7 position of guanine [6]. This alteration, however, does not seem to be related to carcinogenesis [7]. Loveless [8] pointed out that alkylation of O^6 position of guanine might be an important carcinogenic event since it induces mutations in viruses. Gershman and Ludlum [9] did show, since then, that a synthetic polymer of poly C and O^6 methyl guanine induces misincorporation by RNA polymerase.

DMNA induces other important lesions in hepatocytes, mainly protein synthesis inhibition [10], polyribosome disaggregation [11]. RNA polymerase activity is inhibited as well [12].

DNA synthesis is a moment of high sensitivity to carcinogenic effect of DMNA since one single dose of 9 mg/kg of the drug administered during that period induces liver tumours [2].

However, the lesion(s) responsible for the development of cancer is still unclear as is the relationship between the effect of DMNA on DNA synthesis and the other biochemical lesions brought about in the regenerating liver.

Given during the prereplicative period (6 hr after partial hepatectomy) DMNA prevents the increase of the DNA polymerase α activity. [13], and the first DNA synthesis wave [14]. On the other hand, the same dose administered during replicative period (20 hr after partial hepatectomy), only slightly inhibits DNA polymerase α activity [13] while deeply altering DNA synthesis [14].

However, these experiments were not carried out beyond the first replication wave, when regeneration is far from being completed. Little

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is known on how long DNA synthesis is inhibited.

We investigated the effect of 9 mg/kg DMNA given either soon after partial hepatectomy (30 min) or 24 hr thereafter, on DNA synthesis during the first 2 weeks after operation. Moreover, we compared the DMNA-dose dependence of DNA synthesis inhibition when 4 different doses of the drug were administered 30 min or 24 hr after partial hepatectomy.

Our results indicate that DNA synthesis starts to resume only 56 hr after partial hepatectomy when 9 mg/kg DMNA are given immediately after partial hepatectomy, and 96 hr thereafter if the drug is administered 24 hr after surgery. During 168 hr of investigation, DNA synthesis never reaches the peak control values.

On the other hand, 2, 4, 9 or 18 mg/kg DMNA inhibits DNA synthesis to a similar extent whether given 30 min or 24 hr after partial hepatectomy, suggesting thus the preponderance of template alteration on direct effect on the enzymes in the observed inhibition.

MATERIAL AND METHODS

Chemicals

Dimethylnitrosamine was purchased from Merck (Darmstadt) and kept at −20°C in the dark. Dilutions are made with 0.15 M NaCl immediately before use. Thymidine-6[^{3}H] (specific activity 10 Ci/mmole) was purchased from IRE (Belgium).

Treatment of animals

Female Wistar rats weighing 150–170 g at the time of treatment were used.

Experiments summarized in Fig. 1 were performed with animals from our breeding colony. All the other experiments were made with animals from the same strain, but purchased from Janssen Pharmaceutica (Belgium). Partial hepatectomies, according to the technique of Higgins and Anderson [15] were performed by the same investigator between 9 and 12 a.m. At the moment of surgery, animals were starved since 18 hr. Each experimental group was made of 3–8 rats and treated in parallel to a control group of a similar size. The rats were injected i.p. with 20 μCi ^{3}H-thymidine and sacrificed 1 hr later by stunning and bleeding. The times of sacrifice are mentioned in the text.

Extraction of DNA

The livers, perfused *in situ* with 40 ml ice-cold 0.25 M sucrose through the portal vein, were quickly excised, minced with scissors and homogenized in 7 volumes 0.25 M sucrose 1.8 mM $CaCl_2$ in a Potter tube. Nuclei were collected in the pellet after a 600 × ***g***, centrifugation (10 min) at 4°C and purified by spinning in 2.2 M sucrose 1.8 mM $CaCl_2$ according to the method of Chauveau *et al.* [16].

DNA was extracted by the Schmidt–Tannhauser method [17]. The radioactivity was measured in a Nuclear–Chicago Mark II liquid

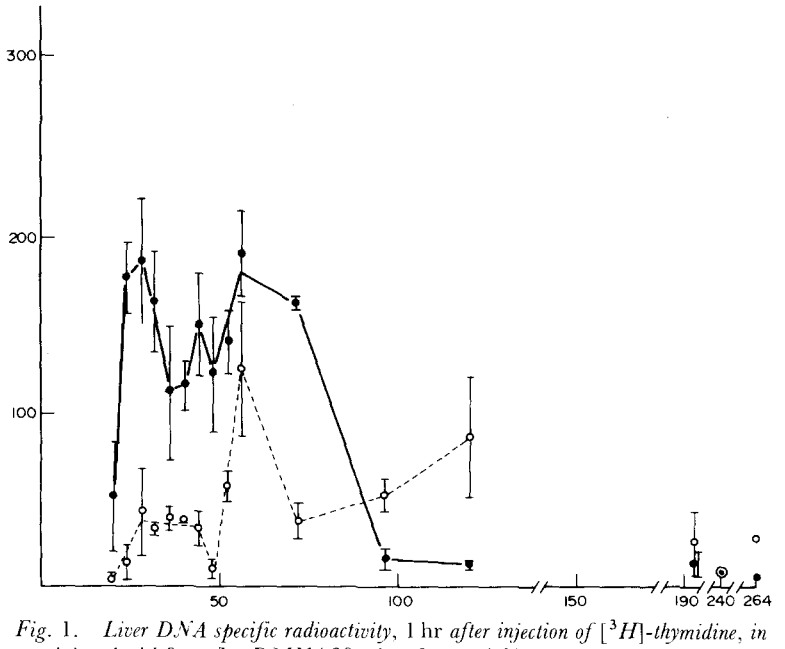

Fig. 1. Liver DNA specific radioactivity, 1 hr after injection of [^{3}H]-thymidine, in rats injected with 9 mg/kg DMNA 30 min after partial hepatectomy.
Abscissa: time of sacrifice after partial hepatectomy.
Ordinate: specific radioactivity in 10^3 dis/min/mg DNA.
(values ± S.E.).

scintillation counter with Instagel (Packard) ready-made scintillation cocktail. Quench corrections were made by external standard method. DNA concentration was estimated by the Burton's modification of the Dische colorimetric reaction [18].

Estimation of acid-soluble radioactivity

A 10-ml aliquot of the first low-speed centrifugation supernatant was precipitated with perchloric acid (final concentration 0.5 N). After allowing the supernatant to stand for 30 min in the cold, it was then collected by centrifugation. The pellet was washed once with 5 ml 0.5 N perchloric acid, and centrifuged. Both supernatants were combined and radioactivity counted as above.

Statistical analyses

Student's *t*-test and standard deviation were calculated with the Hewlett Packard 25 program.

RESULTS

The effects of 9 mg/kg DMNA administered 30 min or 24 hr after partial hepatectomy are summarized in Figs. 1 and 2.

In both sets of experiments, 3 DNA synthesis peaks can be clearly seen in control animals. The relative importance of these peaks is different owing probably to the different origin of the rats (see Material and Methods).

This is consistent with other published data. For instance, Hwang *et al.* [19] found that DNA is synthesized in two waves until the 50th hr after partial hepatectomy. In our case, however, the first peak is somewhat delayed (28 hr after surgery) compared with Hwang's (20 hr).

Given soon after partial hepatectomy, 9 mg/kg DMNA dramatically inhibits the first two DNA synthesis waves (Fig. 1). Tritiated thymidine is not incorporated to an important extent in liver DNA before the 56th hr following surgery. The overall regeneration process is delayed since 120 hr after surgery exogenous thymidine incorporation is much higher in DMNA treated rats while in control rats it reaches values close to those of unoperated animals.

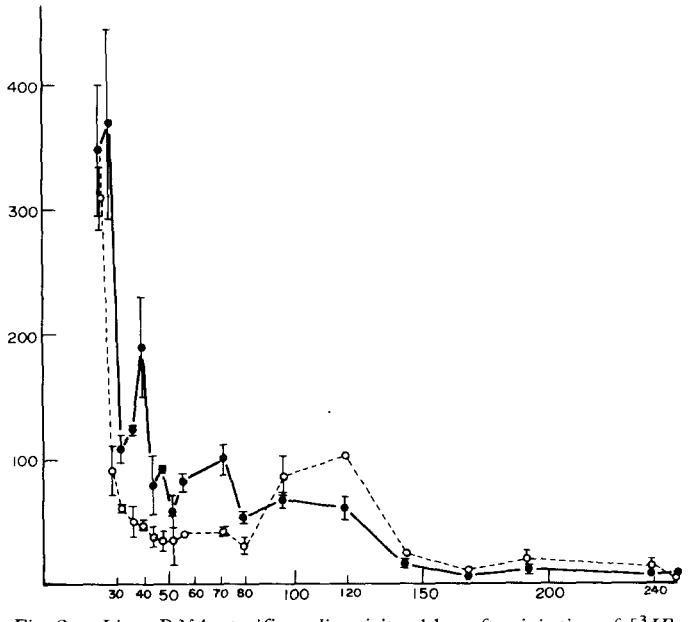

Fig. 2. Liver DNA specific radioactivity, 1 hr *after injection of* [^3H]-*thymidine, in rats injected with* 9 mg/kg *DMNA* 24 hr *after partial hepatectomy.*

Abscissa: time of sacrifice after partial hepatectomy.
Ordinate: specific radioactivity in 10^3 dis/min/mg *DNA.*
(*values* $\pm$ *S.E.*)

Administered 24 hr after partial hepatectomy, 9 mg/kg DMNA quickly inhibits DNA synthesis, which resumes only 96 hr after operation (72 hr after administration of the drug) (Fig. 2).

Our results are in good agreement with those of Craddock [14] with regard to both the deep inhibition of DNA synthesis when the drug is administered early after partial hepatectomy, and the speed of this inhibition when DMNA is administered during S phase. Moreover our results show that in both experimental schemes, DNA inhibition is a long lasting phenomenon, disturbing the whole liver regeneration.

Recently, Herzog and Farber [12] showed that DMNA inhibits RNA synthesis by acting

directly on RNA polymerase rather than on the DNA itself.

In order to see whether inhibition of DNA synthesis also results from enzyme inhibition, we compared the effect of 4 different doses of DMNA (2, 4, 9, 18 mg/kg) but given 0.5 or 24 hr after partial hepatectomy, on DNA specific activity 28 hr after surgery.

We chose this moment since, in both experimental conditions, this is the point of maximal difference between DMNA-treated and control animals.

The reason why we decided to check the inhibition mechanism by this rather indirect way is the following. The enzymes necessary for DNA synthesis are present at a very low level in normal adult rat liver. During regeneration following partial hepatectomy, their activity increases at well determined moments [19].

By giving the carcinogen at 0.5 hr after partial hepatectomy, we avoid direct interaction of the drug with the enzyme molecules, while such an action is produced when the drug is administered 24 hr after partial hepatectomy.

On the other hand, DNA alkylation extent in N7 position of guanine increases linearly with DMNA dose at least in normal liver [20]. Moreover, guanine is alkylated in N7 position in regenerating liver DNA to a similar extent whether DMNA is given 6 or 24 hr after partial hepatectomy [14]. Alkylation in N7 position of guanine is simply indicative of the actual DMNA metabolism.

Thus, if DNA synthesis is inhibited because of direct action on proteins involved in this process, DMNA should be more effective when given 24 hr after surgery when the enzyme is present in high concentration [19].

Results summarized in Fig. 3 show that in fact the drug inhibits thymidine incorporation in DNA to a similar extent when given immediately after partial hepatectomy or 24 hr thereafter. The only exception is the 2 mg/kg DMNA dose, which is effective only if given immediately after surgery. This is the only point where the difference between the two timings is statistically significant (Table 1).

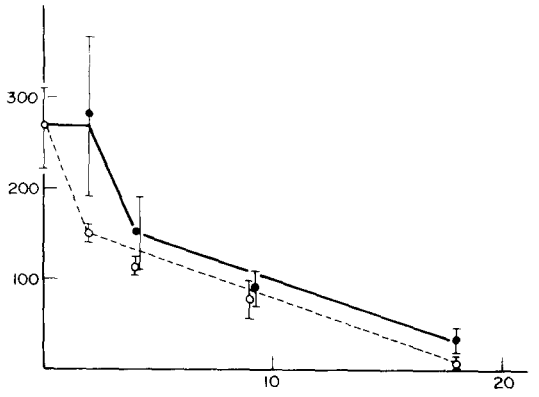

Fig. 3. Liver DNA specific radioactivity, 1 hr after injection of [^3H]-thymidine, in rats injected with different doses of DMNA either 30 min or 24 hr after partial hepatectomy and killed 28 hr after surgery.

Abscissa: dose of DMNA injected.
Ordinate: specific radioactivity in 10^3 dis/min/mg DNA. (values ± S.E.)
Full line: DMNA injected at 24 hr.
Dotted line: DMNA injected at 30 min.

DNA synthesis inhibition is not linked linearly to the DMNA dose. Clearly it is a double component function. Neither direct effect on template nor on enzymes alone can account for such a graph (Fig. 3). [^{3}H]-thymidine incorporation in DNA does not seem to be impaired by a mere unavailability of the radioactive precursor since acid-soluble radioactivity accumulates in supernatant while DNA specific activity decreases (Fig. 4). Here again, there is no statistical difference between acid-soluble radioactivity in supernatant when DMNA is given shortly or 24 hr after surgery (Table 2).

The coincidence between the results of Tables 1 and 2 cannot be understood by assuming that DMNA at 0.5 hr would inhibit enzyme synthesis while at 24 hr it would alter the enzyme molecules already present in the cell.

Table 1. DNA specific radioactivity in rats injected with different doses of DMNA 30 min or 24 hr after partial hepatectomy and sacrificed 28 hr after surgery

DMNA dose injected	Time of DMNA administration		*t*-test			
	30 min	24 hr	*t*	Degrees of freedom	*P*	Commentary
2 mg/kg	147 ± 11	223 ± 18	5.78	2	$0.02 < P < 0.05$	Significant
4 mg/kg	115 ± 11	147 ± 40	1.38	4	$0.2 < P < 0.3$	Non significant
9 mg/kg	71 ± 22	92 ± 20	0.89	4	$0.3 < P < 0.5$	Non significant
18 mg/kg	12 ± 7	33 ± 13	2.02	4	$0.1 < P < 0.2$	Non significant
Controls	266 ± 48					

[^{3}H]-thymidine is injected 1 hr before sacrifice. Values in 10^3 dis/min/mg DNA ± S.E.

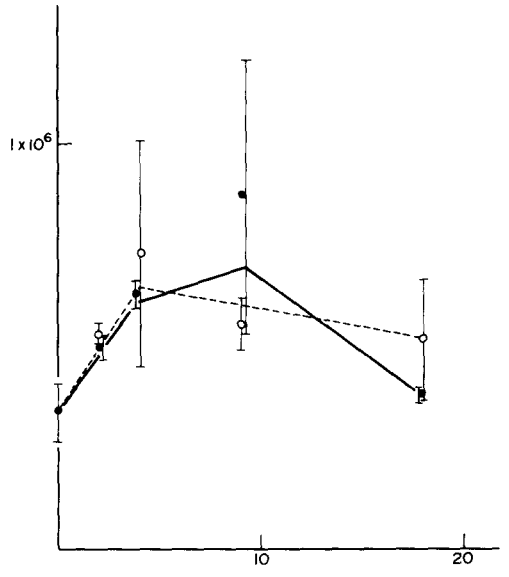

Fig. 4. Acid-soluble radioactivity in the low speed supernatant fraction of the livers used for DNA analysis in Fig. 3.
Abscissa: dose of DMNA injected.
Ordinate: radioactivity in dis/min/g *wet weight of liver. (values ± S.E.)*
Full line: DMNA injected 24 hr *after partial hepatectomy.*
Dotted line: DMNA injected at 30 min.

when the same dose of DMNA was given 0.5 hr after partial hepatectomy.

It should be pointed out that thymidine kinase activity cannot be the limiting factor in exogenous [^{3}H]-thymidine incorporation into DNA. Indeed, we calculated that the lowest level of enzyme activity, measured *in vitro* after DMNA administration is still sufficient to produce at least 50 times more TMP than is actually incorporated into DNA. DNA-polymerase [13], but not thymidine–kinase [21], could be the limiting factor in [^{3}H]-thymidine incorporation in DNA when the carcinogen is administered during the early replication period (0.5 hr). On the contrary, even the highest DMNA dose (18 mg/kg) given 24 hr after partial hepatectomy failed to inhibit thymidine kinase activity measured 24 hr later [21]. DNA-polymerase activity was not inhibited by 9 mg/kg DMNA administered 24 hr after surgery [22]; at that moment, however, [^{3}H]-thymidine incorporation in regenerating liver DNA is inhibited by the same DMNA dose.

Nevertheless, in carcinogen-treated animals, [^{3}H]-thymidine incorporation into regenerating liver DNA is always at least 20 times higher than in normal, unoperated animals.

Table 2. Acid-soluble radioactivity of liver supernatants from rats injected with different doses of DMNA 30 min *or* 24 hr *after partial hepatectomy and sacrificed* 28 hr *after surgery*

DMNA dose injected	Time of DMNA administration		*t*-test			
	30 min	24 hr	*t*	Degrees of freedom	*P*	Commentary
2 mg/kg	531 ± 23	497 ± 27	1.47	3	$0.2 < P < 0.3$	Non significant
4 mg/kg	726 ± 284	627 ± 22	0.47	4	$0.5 < P < 0.9$	Non significant
9 mg/kg	554 ± 64	874 ± 340	0.26	4	$0.5 < P < 0.9$	Non significant
18 mg/kg	521 ± 146	384 ± 21	1.73	3	$0.1 < P < 0.2$	Non significant
Controls	336 ± 71					

[^{3}H]-thymidine is injected 1 hr before sacrifice. Values in 10^3 dis/min/g liver ± S.E.

DISCUSSION

Exogenous thymidine incorporation into DNA after partial hepatectomy depends on the increase in thymidine—and thymidylic kinase and in DNA polymerase activities. Clearly, DMNA can affect both enzymes activities.

Salisbury and O'Connor [13] showed that DNA polymerase activity measured 24 hr after partial hepatectomy is inhibited when 9 mg/kg DMNA are given 6 or 12 hr after surgery, but the inhibition is much less important when the same dose is administered 20 hr after operation.

Similarly, Gol-Winkler and Goutier [21] found that thymidine kinase activity is inhibited

It is not yet clear whether some particular cell types keep incorporating [^{3}H]-thymidine into liver DNA and what type of replication pattern might be assigned to this residual incorporation.

Lee and Spencer [23] reported that, after [^{3}H]-DMNA administration to pregnant rats, liver RNA is methylated to different extents in the different liver lobes. Thus, some cells might undergo less damage and keep on synthesizing their DNA.

This hypothesis, however, is not confirmed by other experimental results. Ord [24] showed that after treatment with methylnitrosourea, [^{3}H]-

thymidine incorporation into Amoeba proteus DNA continues after low alkylating doses. Recently, De Paermentier *et al.* [25] reported that after DMNA administration to rats 24 hr after partial hepatectomy, DNA synthesis seemed to continue in nuclei which were in S phase at the time of treatment, but that no new nuclei entered the S phase. Rajalakshmi and Sarma [26] showed that, in regenerating rat liver, [^{3}H]-thymidine could be incorporated in replicating DNA, when the parental strands were alkylated.

On the other hand, the residual [^{3}H]-thymidine incorporation in DNA might reflect "repair synthesis". It has been claimed that, after treatment of rats or cultured HeLa cells with alkylating agents, [^{3}H]-thymidine incorporation into DNA could be accounted for by repair replication.

It appears, therefore, that DMNA-induced inhibition of [^{3}H]-thymidine incorporation in DNA is a complex phenomenon. When the drug is administered during the early prereplicative stages, the persistence of lesions in DNA as well as impairment of enzymes induction necessary for DNA replication could account for the observed inhibition.

When the drug is given during DNA replication, enzymes such as thymidine kinase [21] and DNA-polymerase [22] are not affected. The possibility remains, however, that other enzymes involved in DNA replication (enzymes for purine and pyrimidine nucleotides synthesis, DNA ligase) or proteins adapting DNA structure to replication process be damaged by the carcinogens.

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Effects of a Gonadotropin-Releasing Hormone (GnRH) Analogue (A-43818) on 7,12-Dimethylbenz(a)anthracene-Induced Rat Mammary Tumors. Histological and Endocrine Studies*

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Abstract — *The effect of A-43818 [D-leu^6 (des-gly-NH$_2^{10}$, pro-ethylamide^9)]-gonadotropin releasing hormone (GnRH) was investigated on rats bearing 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors. Tumor growth was significantly inhibited by the s.c. administration of 10 μg A-43818, twice daily, for six weeks. A dose of 25 μg seemed less effective. Controls and experimental groups were subjected to radioimmunoassays (RIA) of serum luteinizing hormone (LH), follicle-stimulating hormone (FSH) and prolactin (PRL), and to histological examination of pituitaries and ovaries. Treatment with A-43818 resulted in atrophy of pituitary lactotropes and decreased prolactin concentration in plasma. Plasma LH levels were enhanced whereas FSH levels remained unchanged. The endocrine mechanisms of inhibition of tumor growth are discussed in the light of the well-known hormone dependance of DMBA-induced mammary tumors.*

INTRODUCTION

PURIFICATION and synthesis of the decapeptide gonadotrophin-releasing hormone (GnRH) led to the discovery of a single neurohormone endowed with the ability to promote synthesis and release of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [1–4]. Attempts were then made to synthesize analogues of this molecule, in order to obtain more potent stimulators of gonadotrophin production [5–10] or, conversely, competition antagonists [11–13]. A-43818 is one of these analogues. Depending on experimental circumstances, it is able to stimulate [5, 6, 9, 10] or to inhibit [12] gonadotrophin functions. This component is devoid of toxic side-effects and its action is reversible on cessation of treatment [12].

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Investigation of its potential effect on hormone-dependent tumors was therefore of interest. The present paper describes the inhibitory effect of A-43818 on growth of DMBA-induced mammary tumors and studies the underlying endocrine mechanisms. The anti-tumor effect of A-43818 in the rat has previously been reported as short notes independently by us [14] and by others [15, 16].

MATERIAL AND METHODS

Animals and carcinogen

Mammary tumors were induced in Sprague–Dawley female rats, bred in our laboratory, by a single feeding of 20 mg of DMBA (Eastman Organic chemicals, Rochester, N.Y.) dissolved in sesame oil, at 50 days of age [17].

Tumor size was measured by means of a caliper and expressed as "surface" by multiplying 2 perpendicular diameters. Total tumor surface per rat designates the sum of the surfaces of individual tumors [18].

Fifty-two tumor-bearing rats were housed in colony cages in a temperature – (25 ± 1°C) and light-controlled room (14 hr of light and 10 hr of dark). They had received the carcinogenic dose of DMBA 3 months earlier. They were block-randomized into 4 equal groups, the blocks being made up on the basis of equal number and size of the tumors. Group A served as control. Group B was subjected to ovariectomy. Groups A and B received subcutaneous (s.c.) injections of drug-free solvent. Groups C and D were given 2 injections daily of respectively 10 μg and 25 μg of A-43818 dissolved in 0.5 ml of solvent (0.1% bovine serum albumine–0.9% sodium chloride in water) at 9 a.m. and 5 p.m. Treatments were continued for 6 weeks. Body weights were recorded weekly and vaginal smears were taken daily. Tumors were measured every 2 weeks. Statistical analysis was carried out by the Wilcoxon test on paired differences.

At the end of a 6-week period of treatment, the animals were killed by decapitation with a guillotine (Harvard apparatus, Co. Dover, Mass., U.S.A.). Blood was collected and allowed to clot at room temperature. It was kept in the refrigerator (4°C) overnight and then centrifuged at 3000 rev/min for 15 min, at 4°C. The serum was collected and stored in the frozen state (—20°C) until assayed for hormone levels.

Histological procedures

Pituitaries, mammary glands, tumors and ovaries were fixed in Bouin–Hollande sublimate for at least one week. Mammary glands, tumors and ovaries were stained by hematoxylin, eosin and safran.

The microscopical identification of prolactin cells was performed in immunofluorescence using antibodies to ovine prolactin of well-known specificity [19, 20]. The same paraffin sections were processed for immunofluorescence and then for differential staining by Herlant's tetrachrome method [21] as previously described [19, 20].

Radioimmunoassay (RIA)

Serum levels of FSH, LH and PRL were assayed with the RIA kits supplied by NIAMDD and the results were expressed in ng NIAMDD-Rat-FSH-RP 1, ng NIAMDD-Rat-LH RP 1 and ng NIAMDD-Rat-PRL-RP1 standards/ml.

The numerical results were processed for analysis of variance using Fisher F test [22].

RESULTS

The results given in Table 1 show that A-43818 significantly increased the body weight as compared to the control group and that it induced a significant inhibition of tumor growth either after 4 and 6 weeks. Likewise, ovariectomy produced the expected increase in body weight and tumor regression. The latter was significantly more complete than in the A-43818 treated groups (Table 1). The response to treatment was also evaluated on the basis of individual tumors. Table 2 shows the results of such an analysis and that in the A-43818 treatment groups, as in the ovariectomy group, many of the regressions were complete in 6 weeks.

The results, summarized in Table 3, show that A-43818 significantly increased LH blood levels as compared to the controls. Furthermore, in group C, A-43818 induced a significant inhibition of prolactin release. No effect of A-43818 on plasma FSH was recorded under the experimental conditions investigated in this work. As expected, ovariectomy provoked a sharp rise in FSH and LH plasma values. Vaginal smears taken daily showed that the control rats (group A) had normal oestrus cycles while those receiving A-43818 (groups C and D) had prolonged and irregular cycles.

In control animals (group A), the pituitary glands processed for immunofluorescence and Herlant's tetrachrome staining allowed distinct observation of numerous prolactin cells and gonadotropes of normal size. Each ovary contained follicles at different stages of maturation and two or three normal corpora lutea.

In the ovariectomized animals (group B), the expected hypertrophy of gonadotrophic cells was observed, resulting in typical castration cells with prominent Golgi apparatus. The immunofluorescence technique using antiprolactin serum showed no detectable change of prolactin cells.

In group C, A-43818, 10 μg twice daily provoked some hyperplasia of gonadotrophic cells, but to a lesser extent than that observed in the ovariectomized animals. In immunofluorescence prolactin cells were still detectable but were atrophic, with shrunken cytoplasm. In the same animals, the ovaries contained very small and scanty follicles but numerous young corpora lutea (10–15/ovary) with vacuolated luteal cells.

In group C, A-43818, 10 μg twice daily conditions of pituitary and ovaries were comparable to that of the animals of group C, although their differences with the controls (group A) were less evident.

Table 1. Response of tumor-bearing rats to ovariectomy or treatment with A-43818

Treatments	Mean weight of rats (g)			Mean total tumor "surface" per rat (mm^2)						
	Start of exp.	End of exp.	Difference	Start of exp.	2 wks	Diff. vs Start	4 wks	Diff. vs Start	6 wks	Diff. vs Start
A. Controls	263 (13)*	258 (12)	− 5	276 (13)	455 (13)	+179	601 (13)	+325	739 (12)	+463
B. Ovariectomy	260 (13)	315 (13)	+55	253 (13)	82 (13)	−171	31 (13)	−222	15 (13)	−238
C. A-43818 2 × 10 μg per day	247 (13)	284 (12)	+37 (vs *A*, $P<0.01$)	253 (13)	259 (13)	+ 6	184 (13)	− 69 (vs A, $P<0.02$)	179 (12)	− 74 (vs A, $P<0.01$ vs B, $P<0.05$)
D. A-43818 2 × 25 μg per day	263 (13)	307 (10)	+44 (vs A, $P<0.01$)	256 (13)	339 (13)	+ 83	290 (13)	+ 34 (vs A, $P<0.05$)	400 (10)	+144† (vs A, n.s. vs B, $P=0.02$)

*Between parentheses, number of surviving rats.

†Three rats had died, 2 having complete tumor regression, 1 having tumors of unchanged surface since start of therapy.

Statistical analysis by the Wilcoxon test on paired differences.

Table 2. Response of individual tumors to ovariectomy and A-43818 treatment

Group	Dose†	Initial tumors	Tumors responses* Grew	Static	Regressed	New tumors
A. Controls (0.9% NaCl solution)		21	14	5	2	16
B. Ovariectomy		19	0	1	18	0
C. A-43818	2 × 10 μg/day	21	3	5	13	2
D. A-43818	2 × 25 μg/day	20	6	4	10	3

*Comparing tumor area at 6 weeks to pretreatment areas.
†Given s.c. twice daily even during week-end.
Growing increase by at least 50%; regressing decrease by at least 50%; static change less than 50%.

Table 3. Serum levels of gonadotrophins and prolactin in tumor-bearing rats at the end of the 6-weeks experimental period

Treatments	Gonadotrophins levels (means ± S.E.M.) FSH	LH	PRL
A. Controls	509 ± 190	12 ± 5	30 ± 5
B. Ovariectomy	1538 ± 199	399 ± 35	28 ± 8
C. A-43818 2 × 10 μg per day	519 ± 80	119 ± 30	15 ± 4
D. A-43818 2 × 25 μg per day	526 ± 158	63 ± 24	25 ± 4
A vs B	$P<0.001$	$P<0.001$	n.s.
A vs C	n.s.	$P<0.01$	$P<0.05$
A vs C, D	n.s.	$P<0.01$	$P<0.1$
A vs B, C, D	$P<0.001$	$P<0.001$	$0.1<P<0.2$
B vs D	$P<0.001$	$P<0.001$	n.s.
C vs D	n.s.	n.s.	$P<0.1$
C vs B, D	$P<0.001$	$P<0.001$	n.s.

All the tumors studied in this work were subjected to histological controls and proved to be adenocarcinomas.

DISCUSSION

GnRH analogues containing both the D-aminoacid[6] and C-terminal ethylamide modifications are known to produce a powerful and prolonged release of gonadotropins [5, 7] with resultant gonadotropin agonist activity. In contrast, under certain circumstances, inhibitory effects on reproductive functions have been reported [12, 13]. This apparent paradox is probably due to differences in experimental methods with respect to dose ranges and frequencies of administration. On the basis of the known inhibitory effects of A-43818 on the ovaries, it was suggested that its effects on mammary tumors were the results of a temporary "chemical" ovariectomy [16]. Such a simple explanation is not sufficient to account for the inhibition of tumor growth observed in the experiments reported here.

In the conditions of the present work, A-43818 was found to increase LH secretion, to inhibit prolactin cells, and to be apparently devoid of effect on FSH production. Oestrogen secretion was certainly not suppressed for oestrous cycles were still present. The ovariectomized controls differed from the animals treated with A-43818 on production of at least two hormones that could be instrumental in controlling tumor growth: oestradiol and prolactin. Oestrogen production is practically suppressed by removal of the ovaries. On the other hand in the same animals, pituitary lactotropes and serum prolactin levels were not significantly impaired. Normal prolactin levels are sufficient to promote growth of DMBA-induced rat mammary tumors, and tumor regression after ovariectomy may be ascribed to decrease of prolactin secretion as well as to deprivation of ovarian hormones [23]. Therefore it is of interest to notice that the more complete inhibition of prolactin secretion observed in the animals treated with A-43818 resulted in lesser suppression of tumor growth than in the ovariectomized animals. Clearly a direct action of ovarian steroids on tumor tissue should account for the difference. Such view is in good agreement with recent *in vitro* experiments [24].

The reason why A-43818 depresses prolactin production remains to be determined. It is not only by suppression of ovarian hormones that act as physiological stimulators of prolactin production. Indeed, the complete removal of the ovaries resulted in much lesser effect on prolactin cells. It seems thus that A-43818 is endowed with a specific prolactin-inhibiting activity, either by direct action on the anterior pituitary or by an effect through the hypothalamic regulatory mechanism. Such prolactin-suppressing activity is apparently the means by which A-43818 inhibits growth of DMBA-induced rat mammary tumors.

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Interspersion of Cyclophosphamide and BCG in the Treatment of L1210 Leukaemia and Lewis Tumour

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Abstract – *The therapeutic value of interspersing cyclophosphamide (CPM) chemotherapy and BCG immunotherapy was investigated in two tumour models: L1210 leukemia and Lewis solid tumour (LLT). In the case of L1210 leukemia, the antileukemic effect of CPM was enhanced by subsequent BCG administration where a single cycle of combined treatment was applied; treatment by repeated doses of CPM interspersed with BCG was no more effective than CPM chemotherapy alone. In the case of LLT tumour, the effect of one cycle of combined CPM–BCG treatment was not different from CPM administered alone but treatment by repeated doses of CPM interspersed with BCG immunotherapy was less effective than CPM chemotherapy alone. These results indicate that, while the effect of BCG immunotherapy may be favourable or nil when BCG is applied after cell-reducing chemotherapy, it may be nil or unfavourable when applied repeatedly in interspersed chemoimmunotherapy treatments.*

INTRODUCTION

OUR FIRST experiments on active immunotherapy of L1210 murine leukaemia revealed the efficacy of this form of treatment in eradicating the disease only when a small number ($\leqq 10^5$) of neoplastic cells were present [1]. However, subsequent experiments [2] demonstrated that, even when this necessary condition is fulfilled, individuals may respond in three different ways: (a) complete tumor regression; (b) a "plateau" in tumour growth (lasting as long as 60 days) followed by relapse with a new phase of rapid tumour growth; and (c) no perceptible alteration of tumour growth. A cell kinetic study of the "plateau phenomenon" suggested that the number of "new" cells being produced by the tumour is, in this case, equal to the number of cells being destroyed by immunotherapy, so that an equilibrium is maintained [3]. We wondered if, in such cases, chemotherapy interspersed with immunotherapy might reduce the volume of the tumour enough to enable immunotherapy to eradicate the tumour.

Interspersion of chemotherapy and immunotherapy represents a tempting strategy for tumour therapy. Such interspersion has already been used in clinical trials [7]. We are, nevertheless, reluctant to intersperse chemotherapy and immunotherapy, as we previously showed that, while BCG applied after cyclophosphamide (CPM) is able to cure mice carrying L1210 leukaemia not cured by CPM alone, BCG applied before CPM adversely affects the antineoplastic action of the latter, and induces a strong immunodepression, demonstrated by allogeneic skin graft experiments [6]. Thus the experiments reported here were devoted to an investigation of the value of interspersing chemo- and immunotherapy.

Accepted 7 March 1977.

MATERIAL AND METHODS

(a) In experiments using "L1210 leukaemia", the mice were inoculated i.v. with 10^3 live leukaemia cells on day 0. BCG was injected i.v. at the optimal immunostimulating dose of 1 mg/mouse [7]; and CPM was injected i.p. at the suboptimal dose of 80 mg/kg [5]. Mice were given 1, 2 or 3 cycles of CPM–BCG therapy, one cycle essentially being defined as a single treatment with CPM followed 5 days later by a single injection of BCG. Thus CPM was admin-

istered either on day + 1 only, or on days + 1 and + 21, or on days + 1, + 21, and + 41. Similarly, BCG was administered either on day + 6 only, or on days + 6 and + 26 only, or on days + 6, + 26 and + 46.

(b) In experiments using the "Lewis lung tumour (LLT)", which grows as a solid tumour with lung metastases, 2×10^6 live LLT cells were injected intramuscularly (i.m.) on day 0. BCG and CPM treatments were identical with the protocol used for the L1210 experiments, except that mice were given only 1 or 2 cycles of CPM–BCG therapy.

Mortality was recorded daily and autopsies were made. Differences between groups in the same experiment were analysed either by the χ^2 test (L1210 leukaemia) or by Wilcoxon's assigned-rank non-parametric test (LLT). Each group contained 16–20 mice.

RESULTS

The results are presented in Figs. 1 and 2. In the treatment of L1210 leukaemia (Fig. 1), CPM–BCG combination therapy is more efficient than CPM alone only when the leukaemic mice were given a single cycle of treatment. When the animals receive 2 or 3 cycles of combined therapy, their survival does not differ significantly from that of control mice treated with CPM alone.

While one or two injections of CPM are effective in prolonging survival of animals carrying the Lewis tumor (Fig. 2), one or two cycles of combined CPM–BCG therapy are not effective.

DISCUSSION

These observations confirm (a) the effectiveness of BCG applied as an immunotherapy agent

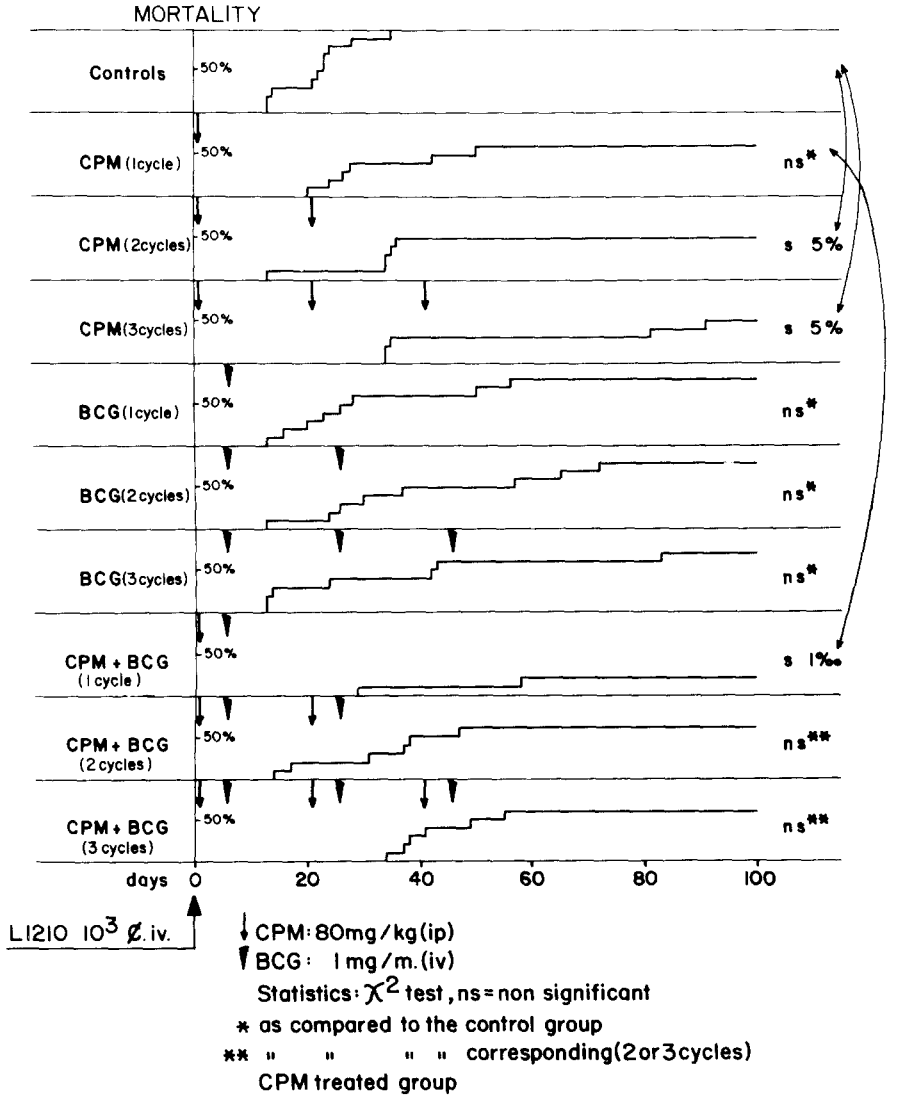

Fig. 1. Interspersion of cyclophosphamide (CPM) and BCG for treatment of L1210 leukaemia: when their sequential combination is given 2–3 times, the effect is poorer than that obtained by one injection of CPM followed by one administration of BCG.

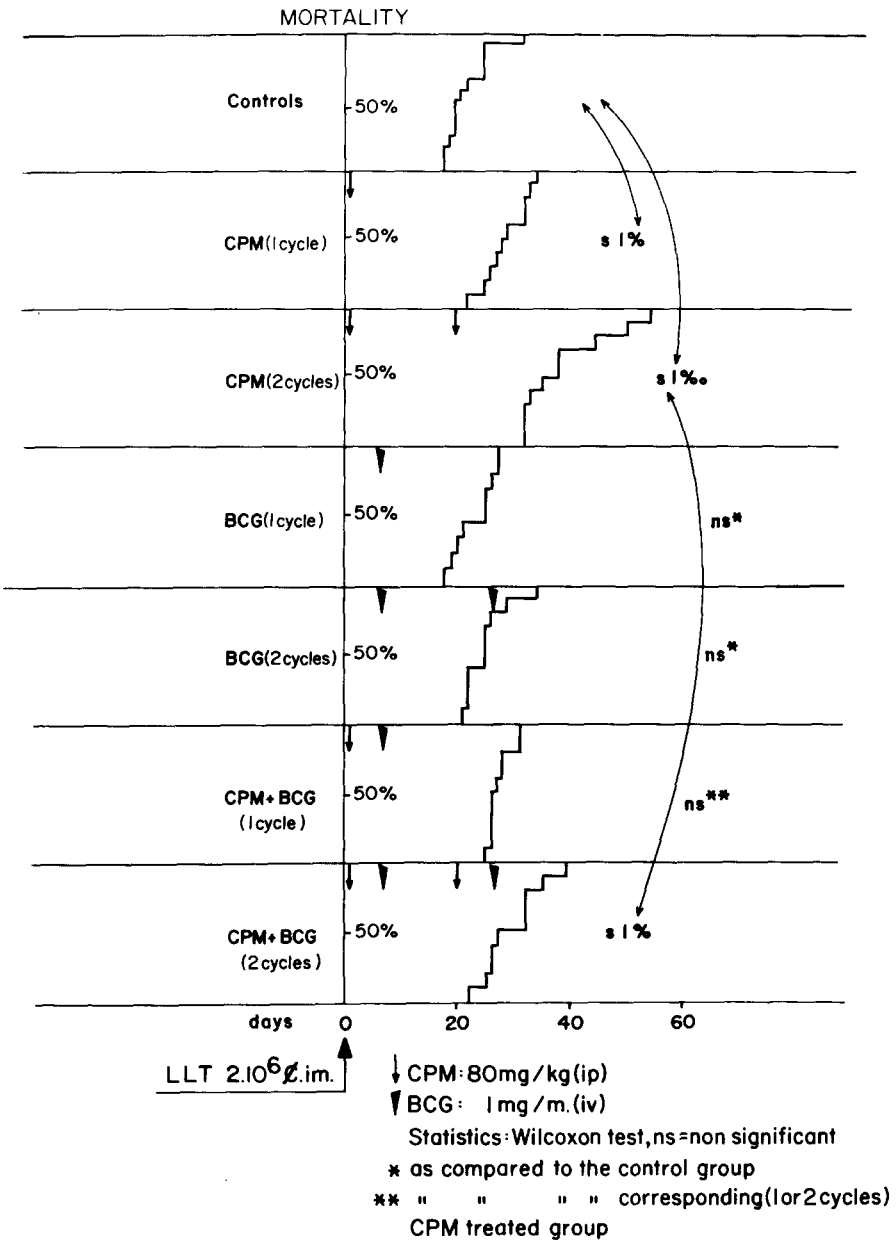

Fig. 2. Interspersion of cyclophosphamide (CPM) and BCG for treatment of Lewis tumour: BCG given after CPM does not improve its effect. When applied twice and interspersed with two injections of CPM, it weakens their effect.

in the treatment of L1210 leukaemia and (b) that BCG is more efficient against this disease when applied after CPM chemotherapy than alone [5], at least with an interval of 5 days.

However, the experiment clearly demonstrates that two or three CPM–BCG sequential combinations are much less effective than one such combination.

As BCG induces a powerful antileukaemic effect when applied after CPM chemotherapy given only once, one is tempted to wonder if the reduced effect of the repeated administrations of both agents is not due to the deteriorative effect of the interspersed sequence: CPM–BCG–CPM. Indeed, we previously showed that the sequence BCG–CPM, when given once, is less effective than BCG or CPM alone [5]. Moreover, we demonstrated that this sequence is a powerful means of inducing immunosuppression as demonstrated by allogeneic skin graft experiments [6].

The effects observed on solid Lewis tumors are more complex to explain. A single application of BCG given after a single injection of CPM is no more active than CPM alone; more interesting is the fact that the mice submitted to two sequences of combined CPM–BCG treatment have a significantly shorter survival than those treated

by two injections of CPM. This may be due either to the same phenomenon as that observed in L1210 leukaemia, namely that BCG applied before CPM adversely affects the effect of the CPM, or to a possible growth enhancing effect of BCG on this tumour, which we have already observed [7].

It should be emphasized that such a result, observed when using an immunosuppressive drug such as CPM, may not be found when using a non-immunosuppressive drug: a later paper will confirm this hypothesis, reporting on an experiment in which the non-immunosuppressive RFCNU [(chloro-2-ethyl)-1-ribofuranosyl-2′-3′ paranitrobenzoate-5′)-3 nitrosourea)] (not published) was used.

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Effect of Synchronization on Chemotherapy of Solid Transplanted Tumours

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Abstract—*A partial synchronization can be induced in the rapidly growing Walker carcinosarcoma and the slowly growing neurosarcoma of the rat by* 6 *applications of hydroxyurea* (50 mg/kg *of body weight) at intervals of* 1.5 hr. *When the block is removed, an increase in the rate of DNA synthesis is observed after* 3–5 hr (^3H-*thymidine incorporation, labelling index*), *which is followed by an increase in the mitotic rate. In order to test whether the effect of therapy with cytostatic agents can be increased by synchronization, different cytostatics were applied at various times after partial synchronization. The times of application were chosen in such a manner that different cell cycle phases were exposed to the action of the cytostatics* (*G*1, *S*, *M*). *The influence of treatment on tumour size, tumour growth and survival time was measured. A strong dose-dependent reduction in tumour growth after cyclophosphamide treatment was similar for both synchronized and non-synchronized animals. Also the growth inhibiting activity of adriamycin was not increased by partial synchronization. This lack of any improvement in the results of therapy using cyclophosphamide and adriamycin is explained by the non-specificity in the mode of action of these substances with regard to the various cell cycle phases. If hydroxyurea, which acts specifically during the S-phase, is used as a therapeutic agent, an improvement in the results of the therapy is obtained by prior synchronization* (1, 3 *and* 4 hr *after partial synchronization*). *Analogous results were obtained with cytosine arabinoside and vincristine, substances which also act predominantly on the S-phase cells.*

INTRODUCTION

THE ACTIVITY of most cytostatic agents varies during the different phases of the cell cycle [1, 2]. In order to improve the effectiveness of the treatment with cytostatics, attempts have been made to increase the proportion of tumour cells in a particularly chemosensitive phase [3–8]. This is achieved by the use of specific inhibitors of macromolecular synthesis. The cells are "collected" at a particular part of the cycle, and after removal of the block reach the sensitive phase synchronously.

Despite promising results obtained after synchronization of both animal and human tumours [4–6, 8–11], it is not yet completely clear whether or not the better therapeutic results really were due to synchronization of the tumour cells [5, 12–18]. In particular, it is difficult to understand why favourable therapeutic results should be obtained after partial synchronization with a substance which does not act specifically during a particular cell cycle phase.

We have now investigated this problem, using rapidly and slowly growing transplanted tumours synchronized with hydroxyurea. Animals were then treated with cytostatic agents cyclophosphamide, adriamycin, hydroxyurea, cytosine arabinoside and vincristine in order to see whether better therapeutic results could be obtained after synchronization.

Accepted 17 February 1977.

We gladly dedicate this paper to Professor Dr. V. Schwartz in celebration of his 70th birthday.

MATERIAL AND METHODS

Animals and tumours

Sprague–Dawley rats (♂, weighing 200 g at the time of tumour transplantation) were maintained under standard conditions (individually in macrolon cages, 25°C, 65% humidity, water and standard diet Altromin^R—from Altrogge, Lage/Lippe—available *ad libitum*). The Walker carcinosarcoma 256 and a solid neurosarcoma were transplanted s.c. in the backs of the animals. The primary tumour of the neurosarcoma was a peripheral tumour of the trigeminus induced transplacentally on the 18th day of

pregnancy by treatment with ethylnitrosourea [19]. Without treatment, animals with the Walker carcinoma survive for 10–12 days, and those with the neurosarcoma *ca.* 10 weeks. In Table 1, data on the cell kinetic parameters of both tumours are presented. The data were calculated from the mitotic indices, labelling indices and percentage of labelled mitoses.

Chemicals

Cyclophosphamide (Endoxan^R, Asta, Brackwede), adriamycin (Adriblastin^R, Farmitalia, Freiburg/Br.), hydroxyurea (HU, m.p. 139°C—we are indebted to Dr. M. Wiessler, Institute for Toxicology and Chemotherapy, German Cancer Research Centre, for the preparation of HU—), cytosine arabinoside (Alexan^R, Mack, Illertissen), vincristine (Vincristin, Lilly, Giessen), ^{3}H-methylthymidine (Fa, NEN, spez. Akt. 20 mCi/mmole).

Toluene scintillator: 34 ml Scintol 3 (Koch–Light Laboratories Ltd., to 1 l toluene.

Chemicals for the preparation of autoradiograms were obtained from Ilford, Essex, England. Other materials were obtained from Merck (Darmstadt).

Synchronization with hydroxyurea

Hydroxyurea (HU) reduces the rate of DNA synthesis to about 5% of the control value at a concentration of 50 mg/kg body weight in both the Walker carcinosarcoma and the neurosarcoma (Fig. 1a). The blockage of DNA-synthesis is carried out by repeated application of HU since the concentration of the reagent in the body rapidly decreases. After the first application, the blockage lasts only 1 hr and after each subsequent application 1.5 hr (Fig. 1b). The following scheme was therefore used: first HU application at 23:30, 2nd application at 0:30 and further applications at intervals of 1.5 hr. In order to determine the maximum possible synchronization with HU, the DNA synthesis was blocked for different times in different groups of animals. Three groups were treated

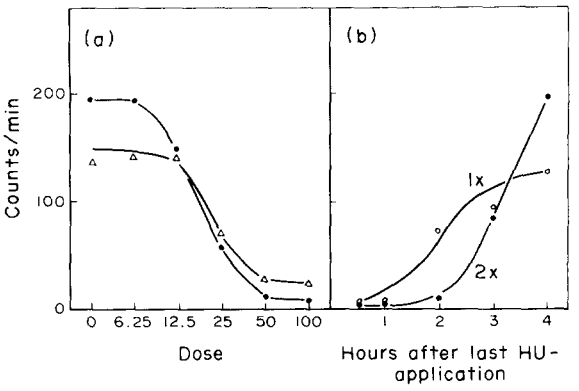

Fig. 1a. Dose–response curve for treatment of the neurosarcoma (△) *and the Walker-carcinosarcoma* (●) *with HU. HU-application (i.p.) at the time* 0. ^{3}*H-thymidine* (0.5 μCi/g *rat, i.p.) was injected* 50 min *later and the animals killed after further* 20 min. *Average values from* 5 *rats* ($n = 60$ *rats*).

Fig. 1b. Comparison of the inhibition in ^{3}*H-thymidine incorporation after* 1 (*time* $t = 0$) *or* 2 (*times* $t = -1$ *and* 0) *injections of HU* (50 mg/kg *body weight*). ^{3}*H-thymidine was injected for a pulse time of* 20 min *and thereafter the animals were killed. Average values from* 6 *rats* ($n = 60$ *rats*).

Ordinates: ^{3}*H-thymidine incorporation per* mg *tumour* (*wet weight*), (△ × 5).

Abscissae: dose (mg HU/kg *body weight*).

with HU either 3, 6 or 9 times, corresponding to a blockage of DNA synthesis for 4, 8.5 or 13 hr respectively. From Fig. 2 it can be seen that the treatment 6 times with HU is sufficient to obtain a good synchronization. The maximal rates of DNA synthesis for the tumour and for normal tissues (duodenum, liver) are reached about 4–6 hr after the last treatment with HU. In all subsequent investigations, synchronization was carried out by 6 successive applications of HU.

Application of cytostatic agents

As soon as the tumours become palpable (Walker-carcinosarcoma, 3–4 days, neurosarcoma, 2–3 weeks), the animals were randomly distributed into groups. After synchronization, the cytostatics were applied i.p. at different cell cycle phases: (1) at the G_1/S border (1 hr after last HU-application), (2) at the maximum in

Table 1. Cell kinetic data on the Walker-carcinosarcoma and neurosarcoma

	LI (%)	MI (%)	t_c	t_s	t_{g2}	t_m	t_{g1}	GF (%)	T_d
Walker-carcinosarcoma	32	3.1	9	5	2.5	0.5	1	58	48
Neurosarcoma	3.6	1.8	22	6	3	3	10	13	192

LI = labelling index, MI = mitotic index, tc = Generation time; t_s, t_{g2}, t_m, t_{g1} = average duration of phase in hr. GF = growth fraction, T_d = time required for doubling of tumour volume in hr.

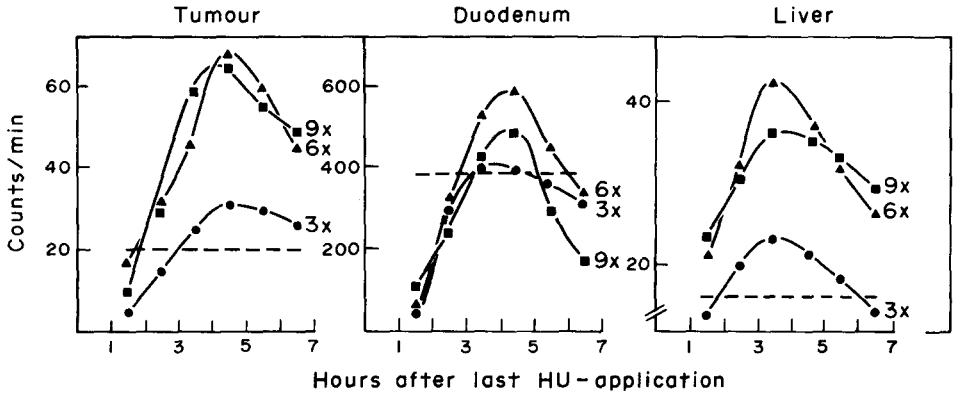

Fig. 2. ^{3}*H-thymidine incorporation in tumour (neurosarcoma), duodenum and liver after partial synchronization with HU (curves), and non-synchronized controls (straight lines). HU was applied (i.p.) in single doses of* 50 mg/kg *body weight (intervals of* 1.5 hr, *time between* 1st *and* 2nd *injections* = 1 hr).
------ *no treatment.*
●——● 3 × HU, *length of block (to time* 0) = 4 hr.
▲——▲ 6 × HU, *length of block* = 8.5 hr.
■——■ 9 × HU, *length of block* = 8.5 hr.
Animals were killed at different times after HU injections (abscissa). ^{3}*H-thymidine was injected* 20 min *previously* (100 μCi/*rat*). *Average values from* 3 *animals at each point* ($n = 60$ *rats*).
Ordinate: counts/min/mg *wet weight.*

DNA synthesis (4 hr after last HU application) and (3) after the decrease in DNA synthesis and entry of the tumour cells into M-phase (8 hr after the last HU application). Non-synchronized groups of animals were also treated with cytostatic agents. Tumour size was determined before and after treatment, using a moveable gauge (two diameters, for the Walker carcinoma every day for 4 days; for the neurosarcoma every 2nd day for 7 days) [18, 20]. In addition to increases in tumour size the survival times were also determined. Different doses (prepared in a geometrical series of dilutions) were used in all experiments, the highest concentration (dose V) corresponding to 4/5 LD_{50}. Six to eight animals were used at each time point and for each concentration.

Liquid scintillation counting

Rates of DNA synthesis were determined by injection of ^{3}H-methylthymidine 20 min prior to killing the rats. After killing, the organs were removed, packed in labelled plastic bags, frozen in liquid nitrogen and stored until further use. After thawing, samples were homogenized (ultraturrax) in distilled ice cold water (1:20). Aliquots (3 × 100 μl) of each sample were pipetted on to round filter paper discs (Whatman, 3 MM, 2.3 cm) which were fixed on polistyrol^R sheets with stainless steel pins. Immediately after pipetting the aliquots, the filter papers were dried in a stream of warm air (60 sec) and extracted in ice cold 5% trichloroacetic acid (TCA) (100 filters in 1 l TCA) twice for 30 min. The filters were then washed with ether/ethanol (1:1, 500 ml, 2 × 30 min), ether (250 ml, 10 min), dried and the acid insoluble radioactivity measured by scintillation counting [21, 22].

Autoradiography

Autoradiograms were prepared according to the method of Schultze [23]. Stripping film (Kodak AR 10) was exposed for a period of 3 weeks. The developed sections were stained with HE and 1000 cells were counted by 2 observers for each tumour. For the determination of labelling index (LI) only cells were counted which had more than 5 grains.

Determination of the mitotic index (MI)

Histological sections were stained with acid haemalaun and 2 persons counted at least 1000 cells each to determine mitotic index.

Statistical analysis

Experimental animals were distributed into individual groups using random numbers. Each group contained 6–8 animals. Days on which measurements were to be made were determined before commencing the experiment. Evaluation of results was carried out by two way variance analysis.

We are very grateful in Professor Dr. H. Immich, Department of Medical Documentation and Statistics, University of Heidelberg, and Professor Dr. E. Weber, Institute for Documentation, Information and Statistics, German Cancer Research Centre, Heidelberg,

for discussions on the statistical evaluation of experimental results.

RESULTS

Synchronization

The degree of synchronization obtained by blockage of DNA synthesis in the Walker-carcinosarcoma and neurosarcoma with HU for 8.5 hr is shown in Figs 3 and 4. When treatment with HU is stopped, a peak in DNA synthesis is observed (^{3}H-thymidine incorporation) which is followed by an increase in mitotic rate. The curves then return to control values. The HU applied during synchronization had no effect on the growth (tumour size) of either tumour.

Therapy

Both the solid Walker-carcinosarcoma and the solid neurosarcoma respond to treatment with cytostatics (Fig. 5). Cyclophosphamide has a very pronounced, dose-dependent inhibitory effect on the growth of the Walker-carcinosarcoma, whereas the effect on the slowly growing neurosarcoma is not so evident. In the following experiments, the tumour size was measured on different days after application of cytostatic agents and the average daily increase was taken as a measure of the success of therapy.

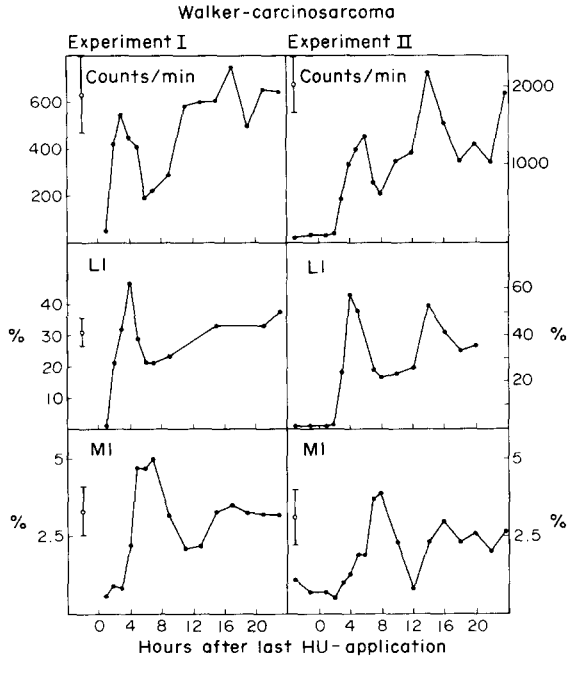

Fig. 3. Effect of 6 applications of HU on solid Walker-carcinosarcoma in two independent experiments on ^{3}H-thymidine incorporation (top, counts/min*), labelling index (middle, LI), and mitotic index (bottom, MI). At each point, 3 (exp. I) or 6 (exp. II) synchronized animals and the same number of non-synchronized control animals were used ($n=278$ rats). At the times shown (abscissa) the animals were killed. ^{3}H-thymidine (exp. I, 0.5 μCi/kg; exp. II: 1 μCi/kg body weight) was injected i.p. 20min (exp. I) or 60 min (exp. II) prior to killing. Each tumour was removed and cut into two parts, one of which was fixed in formalin and used for the determination of LI and MI and the other frozen in liquid nitrogen for measurement of ^{3}H-thymidine incorporation* (counts/min/mg *wet weight). The form of the curves after synchronization is statistically significant in all cases ($P=0.05$). The shape of the curve obtained for the control values was not significant and is therefore presented as one point (with S.D.).*

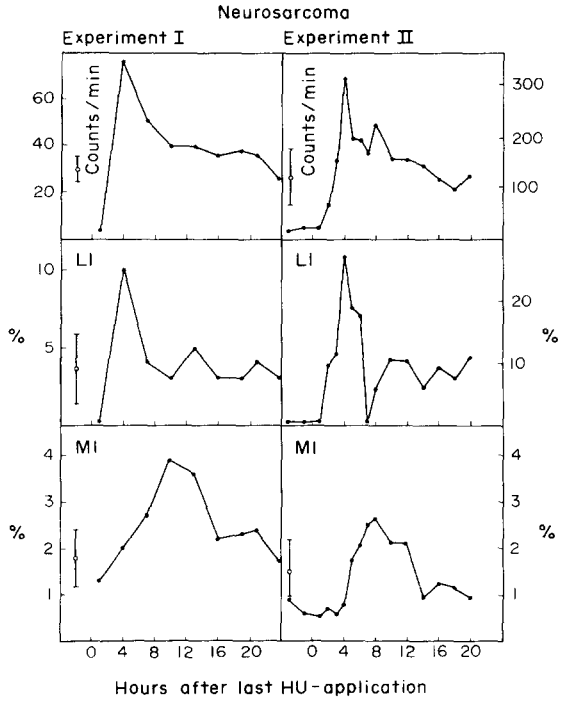

Fig. 4. Partial synchronization of solid neurosarcoma after 6 applications of HU in 2 independant experiments. At each time point, 3 (exp. I) or 7 (exp. II) synchronized animals and the same number of non-synchronized animals were used ($n=306$ rats). For details, see Fig. 3. The form of the curves after synchronization is statistically significant ($P=0.05$). The shape of the curve obtained for the control values was not significant and is therefore presented as one point (with S.D.).

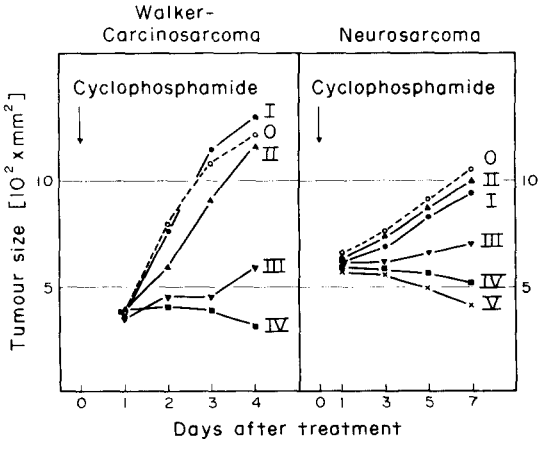

Fig. 5. Growth curve (tumour size in mm^2*) for the Walker-carcinosarcoma and the neurosarcoma without treatment (○) and after application of different doses of cyclophosphamide* (mg/kg: I $=9.5$; II $=19$; III $=38$; IV $=76$; V $=152=4/5$ LD$_{50}$). *Days of application are indicated by arrows. Average values from 6 tumours at each point ($n=66$ rats).*

In order to test whether the efficiency of therapy could be increased by prior synchronization with HU, cytostatics were applied to groups of animals at different times after synchronization with HU. The times of application were chosen in such a way that cells in different phases of the cell cycle were exposed to the cytostatic agents (late G_1, S, mitosis).

The data obtained on the effects of cyclophosphamide on the synchronized and non-synchronized Walker-carcinosarcoma are presented in Table 2 (tumour size, increase in tumour size and survival time). Although a clear dose-dependent reduction in tumour growth and a lengthening of survival times of the animals is obvious in all cyclophosphamide-treated groups, no differences could be observed between the effects on synchronized and non-synchronized animals. The time of application of cyclophosphamide with respect to the last HU treatment did not influence the results.

Analogous results were obtained for the neurosarcoma, in which similar dose-dependent inhibitions of tumour growth were observed for the synchronized and non-synchronized animals (Table 3). The results of therapy were also similar for both cases, and independent of the time of application of cyclophosphamide.

The cytostatic agent adriamycin exerts a much smaller effect than cyclophosphamide on the growth of the neurosarcoma. In contrast to the non-specific action of cyclophosphamide, adriamycin is presumed to affect proliferating cells in the S-, G_2- or M-phases of the cell cycle [1]. For this reason, the possibility of increasing the activity of adriamycin by prior synchronization of the cells was investigated. Adriamycin was injected 1, 4 and 8 hr after the last application of HU. Table 4 shows that the activity is not increased at any time by this procedure.

Other authors reported that cyclophosphamide and adriamycin preferentially affect cells in S-phase [4–6]. We therefore carried out an experiment in which the synthesis of DNA was blocked with HU, cyclophosphamide, or adriamycin, were applied and the block in DNA synthesis maintained for a further 5 hr. A reduction in the effect of the therapy owing to the block in DNA synthesis, which would be expected if the opinion of these authors is correct, was not observed (Table 5).

*Table 2. Tumour size** (1st *number*), *and daily increase†* *of Walker-carcinosarcoma* (2nd *number, italics*), *and survival time of tumour bearing rats* (3rd *number*) *after synchronization and treatment with different doses of cyclophosphamide*

Doses (mg/kg)	Synchronized A = 1 hr	Synchronized B = 4 hr	Synchronized C = 8 hr	Not synchronized D = 4 hr
0	848	1052	854	1066
	294	*346*	*291*	*350*
	7	7	7	7
9.5	1053	1013	904	1130
	377	*331*	*307*	*408*
	8	8	8	8
19	1102	844	729	899
	381	*289*	*195*	*246*
	9	10	9	9
38	655	572	554	491
	164	*171*	*135*	*58*
	12	13	13	13
76‡	227	265	263	412
	3	*13*	*−6*	*−8*
	18	18	17	18

Each point represents the average values from 7 animals ($n = 140$ rats). A–C represents the different times of treatment with cyclophosphamide after the last HU application, group D was treated together with group B.

*On 3rd day after therapy, mm^2.

†Calculated from the values for tumour size on 1st and 3rd days, mm^2.

Analysis of variance:

tumour size: doses = $P \leqq 0.001$; treatment = not significant.

increase: doses = $P \leqq 0.001$; time of application = not significant.

survival time: doses = $P \leq 0.001$; time of application = not significant.

‡ = 2/5 LD_{50}.

The lack of an improvement in therapeutic results, using cyclophosphamide and adriamycin after partial synchronization can be explained by the relatively unspecific action of these substances during the cell cycle. In the subsequent experiments, substances were used which have a more specific mode of action [1, 24]. An improvement in the success of therapy was indeed observed with HU when applied during the maximum in DNA synthesis (3 and 4

Table 3. Tumour size and daily increase of size† (italics) of neurosarcoma after synchronization and treatment with different doses of cyclophosphamide*

Doses (mg/kg)	Synchronized A = 1 hr	Synchronized B = 4 hr	Synchronized C = 8 hr	Not synchronized D = 4 hr
0	956	1036	1048	1095
	70	*84*	*77*	*75*
9.5	942	992	993	891
	67	*63*	*77*	*62*
19	837	1037	870	1094
	52	*69*	*70*	*69*
38	773	618	736	700
	41	*33*	*39*	*32*
76	420	540	499	634
	7	*10*	*14*	*9*
152‡	374	485	360	405
	−9	*11*	*−1*	*−4*

Each point represents the average values from 7 animals ($n = 168$ rats). A–C represents the different times of treatment with cyclophosphamide after last HU application, group D was treated together with group B.

*On 7th day after therapy, mm^2.

†Calculated from the values for tumour size on 1st and 7th days, mm^2.

‡ = 4/5 LD_{50}.

Analysis of variance:

tumour size: doses = $P \leqq 0.001$; treatment = not significant.

increase: doses = $P \leqq 0.001$; treatment = not significant.

Table 4. Tumour size and daily increase of size† (italics) of neurosarcoma after synchronization and treatment with different doses of adriamycin*

Doses (mg/kg)	Synchronized A = 1 hr	Synchronized B = 4 hr	Synchronized C = 8 hr	Not synchronized D = 4 hr
0	1034	1017	1032	1045
	76	*66*	*90*	*82*
0.5	1210	1003	1054	1273
	98	*82*	*92*	*103*
1.0	1089	1105	1027	1107
	85	*92*	*84*	*85*
2.0	923	931	855	1007
	61	*67*	*67*	*76*
4.0	697	849	1006	895
	46	*62*	*67*	*62*
8.0‡	775	771	708	908
	44	*50*	*38*	*51*

Each point represents the average values from 7 animals ($n = 168$ rats). A–C represents the different times of treatment with adriamycin after last HU application, group D was treated together with group B.

*On 7th day after therapy, mm^2.

†Calculated from the values for tumour size on 1st and 7th days, mm^2.

‡ = 4/5 LD_{50}.

Analysis of variance:

tumour size: doses = $P \leqq 0.01$; treatment = not significant.

increase: doses = $P \leqq 0.05$; treatment = not significant.

Table 5. Comparison of tumour increase between synchronous and asynchronous tumours after treatment with different doses of cytostatic agents*

Cytostatic agents	Doses (mg/kg)	Neurosarcoma synchr.	Neurosarcoma asynchr.	Walker-carcinoma synchr.	Walker-carcinoma asynchr.
	0	95	86	196	232
Cyclophosphamide	38	55	54	99	137
	76	27	19	30	78
	0	97	103		
Adriamycin	4	73	64		
	8	69	65		

*Time of application: 6 hr *before* last HU application.

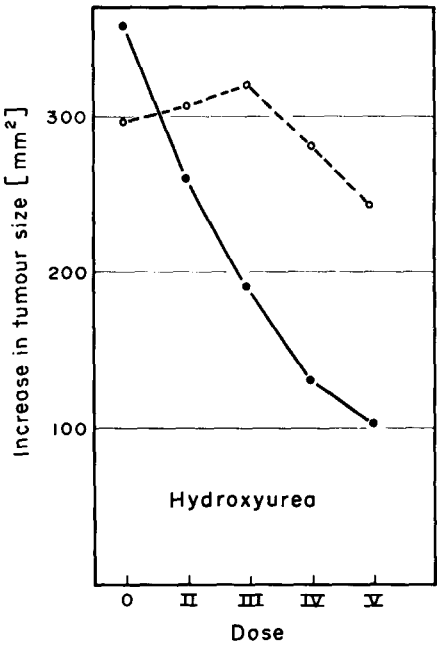

Fig. 6. Increase in tumour size of Walker-carcinosarcoma after synchronization and treatment with different doses of HU. Doses (mg/kg/20 ml *saline*): HU 0 = *control.* II (700), III (1400), IV (2800), V (5600) ●——● *after synchronization* (6 *applications of HU*). ○----○ *not synchronized. Half of the doses given above were applied at the 3rd and 4th* hr *after the last synchronizing HU-injection. Non-synchronized animals were treated at the same times. Average values from* 8 *tumours at each point* ($n = 80$ *rats*). *Statistical analyses: doses:* $P = 0.001$; *synchronized/non-synchronized:* $P = 0.001$.

hr after the last application of HU) for the purposes of synchronization (Fig. 6).

In order to determine whether the improved results with HU can only be obtained when the substance is applied during the maximum in DNA synthesis, therapy was carried out at different times after synchronization. Figure 7 shows that an increased effect is only obtained by injection of HU at 1, 3 and 4 hr after the last HU application. After injection at 8 and 16 hr, no difference could be observed between tumour growth in synchronized and non-synchronized animals.

With this experimental system it can not be said, that the improved therapeutic results are really due to the synchronization or that the pretreatment with HU for synchronization could render the cells more susceptible to a subsequent therapeutic dose following soon after. For this reason, other substances were tested—cytosine arabinoside and vincristine—which have specific prints of attach during the cell cycle. Cytosine arabinoside and vincristine preferentially attack S-phase cells [1, 2] (low doses of vincristine hold cells up in mitosis, whilst 10 times the concentration favours cell kill in the S-phase). The times of application of the cytostatics after synchronization were chosen such that the tumour cells were exposed during the different cell cycle phases. Whereas the asynchronous tumours reacted only weakly or not at all to both substances, synchronized tumours responded clearly to therapy, the major effect being observed after the first two times of application (1 hr = G1-S-phase; 3.5 hr = S-phase) (Fig. 8).

DISCUSSION

In order to study the question whether the success of tumour therapy can be improved by partial synchronization of the tumour cells, experiments were carried out with the solid Walker-carcinosarcoma and neurosarcoma. The tumours were synchronized with HU, a substance which blocks the passage of cells through the S phase but allows cells in G_2, M or G_1 to continue through the cycle until they reach the G_1/S boundary [1, 24, 25]. Using 6 individual applications of HU the synthesis of DNA could be blocked for *ca.* 8.5 hr. With this dose, which corresponds to 1/3 of that used by Rajewsky [24], both tumours could clearly be synchronized. The dose used was chosen so that a total block could be induced in the synthesis of DNA without there being any detectable inhibitory effect on tumour growth.

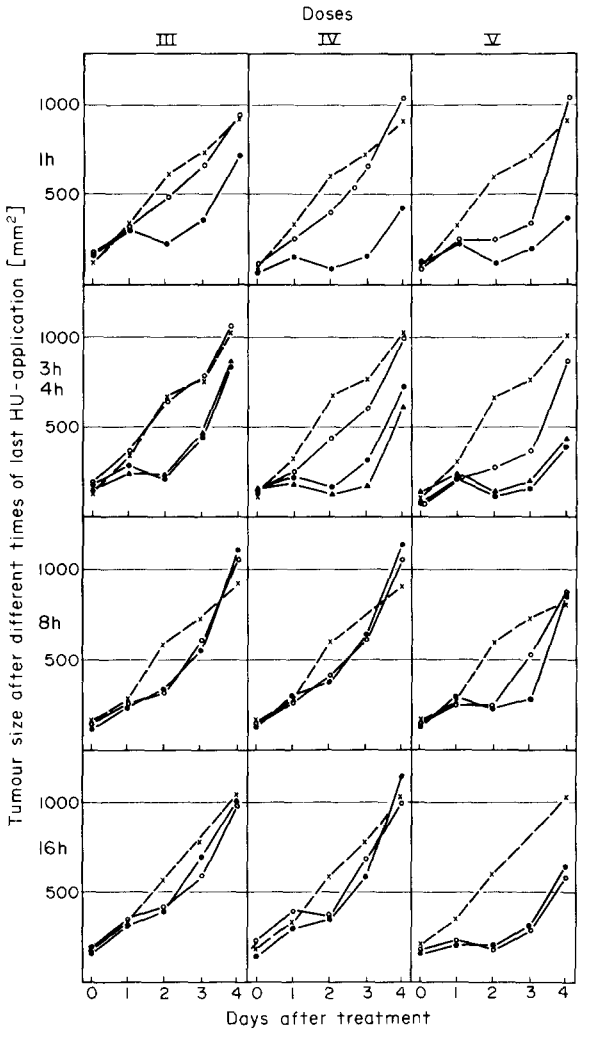

Fig. 7. Tumour size of the Walker-carcinosarcoma on different days after synchronization and treatment with HU. Doses: (mg/kg/20 ml *saline*)*:* III (1400), IV (2800), V (5600). *The therapeutic dose of HU was applied at* 1, 3 *or* 4, 8 *and* 16 hr *after the last synchronizing dose of HU* (*see Table* 1).
× ---- × *non-treated tumours.* ○——○ *treated-non-synchronized tumours.* ●——● *treated-synchronized tumours.* ▲——▲ *tumours treated* 3 hr *after synchronization.*
Average values from 6 *tumours at each point* ($n = 196$ *rats*). *Statistical analysis:*
1 hr*: synchronized/non-synchronized:* $P \leqq 0.001$.
3 hr, 4 hr*: synchronized/non-synchronized:* $P \leqq 0.001$.
8 hr*: synchronized/non-synchronized = n.s.*
16 hr*: synchronized/non-synchronized = n.s.*
For reasons of clarity, the data for synchronized nontherapied animals are presented in the curve for non-treated animals, since synchronized and non-synchronized tumours grow at the same rates.

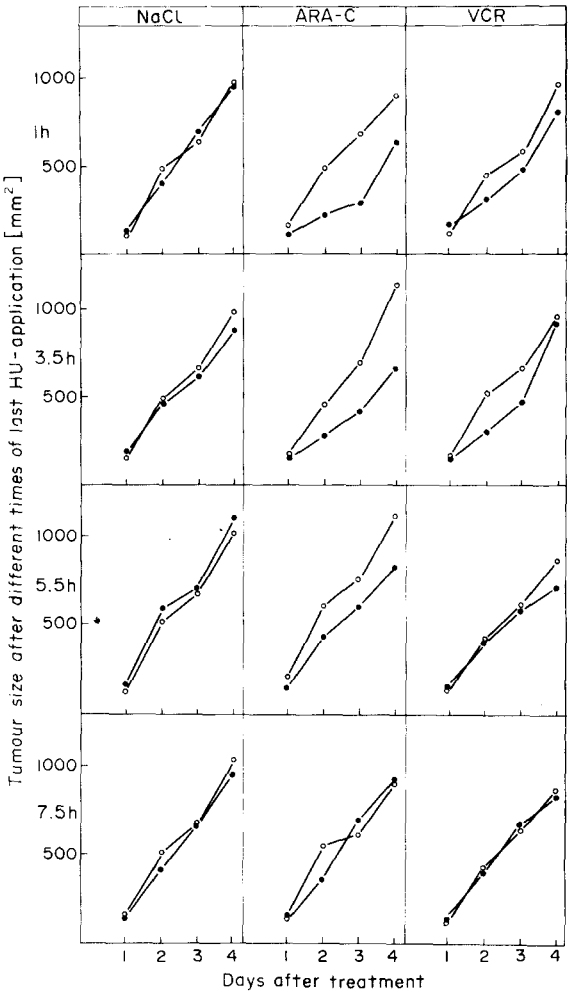

Fig. 8. Tumour size of the Walker-carcinosarcoma on different days after synchronization and treatment with NaCl (control), cytosine-arabinoside (ARA-C) and vincristine (VCR). Doses: ARA-C: 400 mg/kg; *vincristine:* 0.16 mg/kg. *The substance was applied* 1, 3.5, 5.5 *and* 7.5 hr *after the last synchronizing dose of HU.* ○——○ *non-synchronized tumours.* ●——● *synchronized tumours. Average values from* 10 *tumours at each point* ($n = 240$ *rats*). *Statistical analysis:* 1 hr*:* $P \leqq 0.001$*;* 3.5 hr*:* $P \leqq 0.01$*;* 5.5 hr*: n.s.;* 7.5 hr*: n.s.*

In addition to the blockage induced by HU at the G_1/S boundary, it has been reported that HU has cytotoxic effects on cells in the S-phase [1, 24, 25]. The length of the cell cycle for the Walker-carcinosarcoma is relatively short (9 hr). More than half of the cells are in the S-phase at any particular time and can therefore be eliminated by treatment with HU. This explains the fact that in the DNA synthesis peak observed after removal of the block, the control values are not reached. For the slowly growing neurosarcoma (cell cycle duration 22 hr), only 1/4 of the proliferating cells are found in the S-phase and are thus susceptible to the action of HU. When the block is lifted, a maximum in DNA synthesis is reached with values twice as high as in the controls.

Over the past few years, a "synchronization therapy" has been carried out in various clinics. It was hoped that by "collection" of cells in a cell cycle phase which is particularly sensitive to the action of certain cytostatics, the tumour cells could be selectively eliminated.

Various studies have reported favourable therapeutic results with cyclophosphamide after partial synchronization with vincristine [4–6]. This positive effect was interpreted on the basis

of an S-phase specific activity for cyclophosphamide. An apparent contradiction exists here, since other workers have reported that cyclophosphamide has an unspecific effect on all cells [1, 16, 17]. In order to resolve this problem, cyclophosphamide therapy was carried out on two different rapidly growing solid transplanted tumours after partial synchronization. The times of application were chosen such that the cells were exposed to the drug at different phases of the cell cycle (late G_1, S and M). Partial synchronization was carried out with HU rather than vincristine, since using the latter substance, a synchronous passage of the cells through the S-phase can not always be induced [12, 14]. With the tumours used here, dose-dependent increases in the effects of therapy were obtained which were, however, independent of the degree of synchronization (Tables 2, 3, 5). Similarly, at other times after synchronization (3 and 6 hr before last HU-application), no differences were observed. According to these results, cyclophosphamide is not active specifically during one phase of the cell cycle. The improved therapeutic results obtained by other authors using cyclophosphamide after pre-treatment with vincristine could be explained on the basis of a combination therapy with particularly favourable timing of the application of the substances. The pre-treatment with vincristine might lead to a fascilitated drug entry into cells.

Also with adriamycin, which reacts mainly with S/G_2 and M-phase cells, no improvement in results was obtained after synchronization. The results of therapy were the same for synchronized and non-synchronized animals with both the neurosarcoma (Table 4) and the Walker carcinosarcoma (unpublished results). These results do not exclude the possibility that an improvement in the results of therapy would be obtained using an appropriate combination of vincristine and adriamycin [6]. The results do, however, show that no improvement can be obtained after synchronization with adriamycin. This is perhaps not surprising, since the biological half life of adriamycin is about 24 hr [26], and during this time all of the cells can pass through the sensitive cell cycle phases.

If HU is used both as a synchronizing agent and, in higher doses, as a cytostatic substance, a clear increase in the effect of therapy is observed (Fig. 6, 7). This improvement is only obtained if the cytostatic dose of HU is applied at the G_1/S boundary or during the S-phase (1, 3 and 4 hr after partial synchronization). Since the combination of synchronization and therapy, using HU could represent an exceptional case, a further experiment was carried out using cytosine arabinoside and vincristine, which affect predominantly S-phase cells as does HU. The results of this experiment are similar to those obtained using HU. The increased effects of therapy using HU, cytosine arabinoside and vincristine after a synchronization step could be explained by the short biological half lives of these substances which would ensure that each cell cycle phase is exposed invididually to the drug. So, the "synchronization therapy" can improve the effectiveness of tumour therapy with cytostatics.

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The Relationship of Plasma Prolactin to 17-B Oestradiol in Women with Tumours of the Breast

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Abstract – *The concentrations of plasma prolactin and 17-B oestradiol in peripheral venous plasma were measured in 94 female patients previously admitted for breast surgery. The results showed that the levels were not significantly different in those patients with early breast cancer compared with those with benign breast disease. In addition, there was no direct correlation between the circulating levels of oestrogen and prolactin. The levels of prolactin, however, were shown to be significantly lower after the menopause.*

INTRODUCTION

OESTROGEN is thought to be the most important factor regulating the secretion of prolactin in the female rat [1]. This relationship may well be true in women. Friesen and Hwang raised basal prolactin concentrations [2] using pharmacological doses but Jaffe *et al.* were unable to repeat this finding [3]. There seems little doubt that oestrogen potentiates the prolactin response to drug stimulation [4, 5] and more recently it has been shown that relatively low doses of oestrogen are effective in stimulating prolactin secretion [6].

At a more physiological level, the literature is again conflicting. Prolactin levels increase progressively during pregnancy [7] and are elevated in the middle of the menstrual cycle and during the luteal phase [8]. Both these reports suggest that the changes are oestrogen related. However, Hwang *et al.* [9] and Friesen *et al.* [10] found no change in prolactin levels during the menstrual cycle. More recently, Vekemans and Robyn [11] observed that plasma prolactin levels decreased with age and concluded that the fall was due to the declining level of oestrogen. Boyns *et al.* [12] reported a similar finding in patients with breast cancer.

It is well recognised that hormones are involved in the growth and development of breast cancer. Much work has been done on the endocrine status of these patients. In animal work oestrogen, progesterone, cortisol, prolactin and growth hormone have been shown to be necessary for mammary growth and development [13]. However, in women with malignant breast disease, although the plasma concentrations of oestrogen and prolactin have been measured separately, nothing is known about their inter-relationship. The purpose of our study was to investigate this relationship in women with benign and malignant breast disease, by correlating the changes in prolactin with changes in 17-B oestradiol concentration measured on the same sample of blood. We also set out to study the effect of menopausal status on circulating prolactin concentrations in these women.

MATERIAL AND METHODS

Ninety-four patients aged between 25 and 70 yr, who had been admitted for breast surgery were studied. All gave their informed consent. The patients were subsequently divided into two groups: those with benign and malignant disease, according to the histology of the breast tissue removed. Only patients with cancer Stages I and II of the Manchester Classification [14] were included in the group with malignant tumours. Patients were then categorised as being pre- or post-menopausal, depending on whether 6 months had lapsed since the menses had ceased. The menopausal status of these patients was checked 2 yr later, and if any doubt existed about their original classification these patients were excluded from the study. Patients taking drugs

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that might affect oestrogen or prolactin concentrations were also excluded from analysis.

As physical and psychological stress have been implicated in raising prolactin concentrations [15] venepuncture was performed on a visit to the hospital 3 months after surgery. The sample was collected between 9.00 a.m. and 10.00 a.m., because there is evidence of a sleep-mediated 24-hr pattern of prolactin secretion [16]. Plasma was separated by centrifuging at 1000 rev/min for 15 min and immediately stored at −20°C. Prolactin and 17-B oestradiol concentrations were analysed on the same blood sample.

17-B oestradiol was separated on a column of Sephadex LH20 and determined by radioimmunoassay in a system containing the tritiated antigen, and an antiserum to oestradiol-6-carboxymethyloxime-bovine serum albumin [17].

Prolactin was measured by a specific double antibody radioimmunoassay using an anti-human prolactin antiserum raised in rabbits and purified human pituitary prolactin as standard and for iodinisation [18].

RESULTS

The frequency with which the concentrations of prolactin and 17-B oestradiol appeared in each group was studied and it was concluded that in general the distribution was best described by geometric mean and range. The values are shown in Tables 1 and 2. The prolactin results are expressed in micro units of the human pituitary prolactin research standard MRC71/222. An analysis with Students' *t*-test on the log data showed that there were no significant differences in the levels between patients with benign breast disease and those with early breast cancer (Table 1). On application of the Bravais–Pearson coefficient of linear correlation there was no significant tendency to a straight line. The concentrations of prolactin ($P<0.025$) and 17-B oestradiol ($P<0.001$) were significantly higher in the premenopausal, compared with the postmenopausal groups (Table 2).

DISCUSSION

There were no significant differences in plasma 17-B oestradiol and prolactin concentrations between patients with benign breast disease and those with early breast cancer. Data on plasma oestradiol and prolactin concentrations after the menopause is relatively scarce. It is known, however, that plasma 17-B oestradiol production from the ovary ceases abruptly at the menopause [19, 20], and that peripheral venous blood concentrations fall to approximately 80% of their premenopausal values. When comparing prolactin and 17-B oestradiol concentrations in the benign and cancer patients we therefore regarded the postmenopausal group as being homogeneous.

Table 1. *The concentrations of plasma oestradiol and prolactin in patients with benign and malignant disease of the breast*

Plasma oestradiol						
Menopausal status	Diagnosis	Number of patients	Mean (log)	Geometric mean (pmole/l)	Standard deviation (log)	Range (pmole/l)
Premenopausal	Benign	42	2.4159	260.6	0.323	43.1–876.6
	Cancer	16	2.3072	202.9	0.390	34.2–893.9
Postmenopausal	Benign	11	2.0829	121.0	0.370	22.1–293.3
	Cancer	25	2.1128	129.7	0.176	57.0–243.2
Plasma prolactin				(mu/l)		(mu/l)
Premenopausal	Benign	40	2.4128	258.7	0.133	144.0–478.0
	Cancer	14	2.4073	255.5	0.176	132.0–540.0
Postmenopausal	Benign	10	2.2948	197.2	0.200	94.0–372.0
	Cancer	19	2.3439	220.7	0.132	124.0–370.0

Our results agree with other groups who have studied serum prolactin levels in breast cancer [12, 21–24]. However, some of these studies must be interpreted with caution: blood samples were not always taken at a uniform time of day, a variety of control groups was used and data on patients' use of drugs was omitted. Operative stress may also, in some cases, have affected results. Kwa *et al.* [25] suggest that although they found no difference in plasma prolactin concentrations between patients with breast cancer and matched hospital controls, it may be a factor in certain high risk populations.

Owing to the great individual variation in 17-B oestradiol during the menstrual cycle, it is not

Table 2. The concentration of plasma oestradiol and prolactin in pre- and postmenopausal patients with breast disease

Menopausal status	Number of patients	Mean (log)	Geometric mean (pmole/l)	Standard deviation (log)	Range (pmole/l)	*t*. Value	Degrees of freedom	2-tailed probability
Plasma oestradiol								
Premenopausal	58	2.3859	243.2	0.343	43.1–893.9	4.63	90	$P<0.001$
Postmenopausal	36	2.1037	127.0	0.246	22.1–293.3			
			(mu/l)		(mu/l)			
Plasma prolactin								
Premenopausal	54	2.4114	257.9	0.144	132.0–540.0	2.47	81	$P<0.025$
Postmenopausal	29	2.3269	212.3	0.157	94.0–372.0			

justified to comment on our premenopausal data on this hormone. However, postmenopausally there was no significant difference in 17-B oestradiol concentrations between patients with benign breast disease and those with early breast cancer. The only other study we are aware of measuring postmenopausal 17-B oestradiol concentrations in women with breast cancer [26] reported raised concentrations which is in conflict with our results.

Our results show that both prolactin ($P<0.025$) and 17-B oestradiol ($P<0.001$) concentrations were significantly lower in the postmenopausal compared with the premenopausal groups, but there was no correlation between prolactin and 17-B oestradiol concentrations in the same blood sample in either group. In the premenopausal group it could be said that no correlation was shown as 17-B oestradiol concentrations fluctuate greatly throughout the menstrual cycle. However the same cannot be said postmenopausally, when oestrogen levels are relatively constant [20].

We have shown that prolactin levels fall as women with breast disease pass through the menopause, and our results agree with those of Vekemans and Robyn [11] who studied a normal population. However, at physiological levels, in our patients, it would seem that there is no definite direct relationship between prolactin and 17-B oestradiol. This could mean that patients with breast disease differ in the way that prolactin secretion is controlled from "normal" women, or that they form one population in this respect, and prolactin levels in normal women although related to menopausal status or advancing age, are not related to circulating plasma 17-B oestradiol. It must be said that although 17-B oestradiol is the most potent oestrogen and oestriol is only present in much reduced levels, oestrone may well have a greater affinity for hypothalamic receptor sites than 17-B oestradiol and play the major role in control of prolactin secretion. Further investigation into the "prolactin–oestrogen" relationship is obviously required before the role played by oestrogens in prolactin secretion is understood.

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Serum Kappa-Casein in Breast Cancer

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Abstract—*Kappa-casein concentrations were measured by radioimmunoassay in serum samples from patients with breast diseases, and from controls, who were healthy volunteers and patients with various non-malignant diseases. Seventeen of three hundred and sixty-one* (4.7%) *samples from controls gave values of greater than* 60 ng/ml. *Sixty-two samples from lactating women gave values ranging from* 136 ng/ml *to* 1700 ng/ml. *Forty-nine of eighty-eight* (55.6%) *samples from patients with metastatic breast cancer and* 21 *of* 53 (39.6%) *samples from patients with cancer localized in the breast and its regional nodes had K-casein concentrations greater than* 60 ng/ml. *Twelve of forty-nine* (19.7%) *patients with treated breast cancer who were clinically tumour-free at the time of sampling, and* 10 *of* 76 (15.2%) *samples from patients with histologically established benign breast disease had increased serum concentrations of Kappa-casein. No correlation was found between casein levels and age, parity and endocrine status.*

INTRODUCTION

SEVERAL workers have suggested that mammary tumours may produce casein. Turkington [1] reported the presence of a casein-like fluid in a rat mammary carcinoma cultured *in vitro*, whilst Young [2] also demonstrated a milk protein in mammary tumours grown *in vitro*, although the antisera he used were not specifically raised to the casein fraction of the milk proteins.

Radioimmunoassay of alpha-lactalbumin has been performed and various reports [3] indicate that this protein is not elevated in the serum of patients with breast cancer. Hendrick and Franchimont [4] however, have reported that a significant number of patients with breast cancer have elevated concentrations of circulating casein. A radioimmunoassay for casein was, therefore, developed to assess the possible value of this protein as a marker of tumour activity in the management of breast cancer patients.

Normal production of casein in lactating women is known to be dependent on circulating prolactin levels [5], but attempts to induce casein production in cultured mouse mammary tumours by increasing the prolactin concentration in the cultured medium have been only partially successful [6]. Prolactin secretion is not constant throughout the day, and single assays of this are of only limited value. Tests involving stimulation of the pituitary gland to maximal or near maximal secretion of prolactin and other anterior pituitary hormones reflect the endocrine status of the patient more accurately than do random assays of the individual hormones. These tests were, therefore, performed on a small number of patients with elevated concentrations of K-casein in their peripheral blood.

MATERIAL AND METHODS

Preparation of casein

Human milk was obtained from the Human Milk Bureau (South Birmingham HMC), and whole casein was prepared by the method of Malpress and Seid-Akhavan [7].

In view of the instability of whole casein after iodination, this was fractionated by the method of Malpress [7] to yield Kappa-casein, and this fraction was used throughout the study for standards, for labelled antigen and for antiserum production. This was stored at 20°C.

Production of antiserum

One milligram of Kappa-casein was injected into 30 intradermal sites on two rabbits, followed by booster injections after 40 days. A further booster injection was made after 4 months. The

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antiserum was absorbed with 20 mg/ml of freeze-dried normal human plasma. The antiserum gave a single line of precipitation when tested on Ouchterlony double diffusion plates against whole casein and Kappa-casein.

Iodination procedure

Iodination was carried out by modification of the standard chloramine T procedure [8]. Ten micrograms of Kappa-casein in 10 μl of 0.05 molar phosphate buffer (pH 7.5) were added to 1 mCi of ^{125}I in 10 μl of 0.05 M phosphate buffer. Twenty-five microliters of a 1 mg/ml solution of chloramine T in 0.05 M phosphate buffer were added to the solution and mixing was allowed to proceed for 30 sec before 100 μl of 0.05 M phosphate buffer containing 120 μg of sodium metabisulphite was added and allowed to mix for 30 sec. Two hundred microliters of a solution containing 20 mg/ml of bovine albumin and 2 mg/ml of potassium iodide were added to the solution and allowed to mix for one minute before separation of the iodinated protein fraction on a 25 cm × 1.5 cm column of sephadex G 150.

Treatment with iodoacetamide [9] before iodination decreased the immunological damage encountered on iodination; and when prepared in this manner, the ^{125}I Kappa-casein was usable for 7 days.

Assay method

Radioimmunoassay was performed by a double antibody method by means of an automatic, dispensing, filtering and counting apparatus, with computer calculation of results [10].

One hundred microlitres of normal serum and 100 μl of 0.05 M phosphate buffer containing known amounts of K-casein were added to polystyrene tubes for calibration standards. One hundred microliters of sample serum and 100 μl of 0.05 M phosphate buffer were dispensed into other tubes. Fifty microlitres of diluted antiserum were added. The antiserum was diluted into phosphate buffer containing 400 mg/ml of ethylene diaminotetracetic acid (EDTA). The initial dilution of antiserum used was one part in 12,500 giving a final dilution of 1 in 87,500. This mixture was incubated at 37°C for 2 hr.

Fifty microlitres of ^{125}I K-casein diluted in 0.05 M phosphate buffer were then added and incubation was allowed to proceed for a further 2 hr at 37°C.

Fifty microlitres of sheep anti-rabbit globulin antiserum were added and incubation continued for 16 hr at 5°C. Separation was by glass fibre discs on an automatic filtration machine described elsewhere [10].

Quality control

Aliquoted pools of sera with known high and low casein concentrations were stored at −20°C and those were incorporated in each assay as quality controls. The within assay coefficient of variation was 2.5%. The between assay coefficient of variation was 18%.

Samples

Serum was collected from patients with known breast diseases, both benign and malignant, at various stages in the course of the disease. Samples, were also obtained from healthy volunteers, from hospital patients with various non-malignant diseases and from lactating women. The serum was separated and stored at −20°C until assayed for K-casein.

Pituitary stimulation test

The patients selected for this test were all under the age of 60 without any cardiovascular abnormality. Samples of blood were collected for assay of glucose, adrenocorticotrophic hormone (ACTH), growth hormone (GH), cortisol, thyroid-stimulating hormone (TSH), prolactin and K-casein. Soluble insulin (0.15 units/kg), 200 μg of thyrotrophin-releasing hormone (TRH) and 100 μg of luteinizing-hormone-releasing hormone (LHRH) were then injected intravenously. Further samples were collected at 15, 30, 45, 60, 90 and 120 min for estimation of glucose, ACTH, GH, cortisol, TSH, prolactin and casein.

RESULTS

The limit of sensitivity of the assay was 300 pg of K-casein per tube (30 ng/ml). Values were not affected by thawing and freezing samples up to five times, nor by the addition of EDTA to the collecting tubes. Since the inhibition curve obtained with our K-casein and antiserum was almost super-imposable upon that obtained by using our K-casein and Hendrick and Franchimont's anti-whole-casein serum, it was concluded that our antiserum was directed against the same determinants. (We are indebted to Professor Franchimont and Dr. Hendrick for the above result.)

Assay of 361 control samples (72 normal subjects and 289 patients with non-malignant diseases) gave 17 results greater than 60 ng/ml. Assay of 62 samples from lactating women gave values in the range 136 ng/ml to 1700 ng/ml.

Of 76 patients with histologically established benign breast diseases, 10 had values in excess of 60 ng/ml. (Fig. 1). Samples from 53 patients with

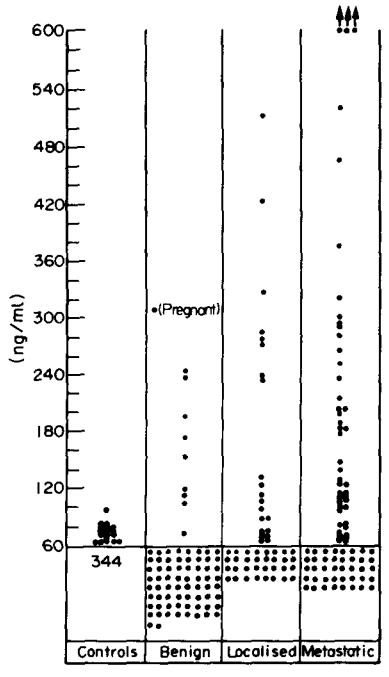


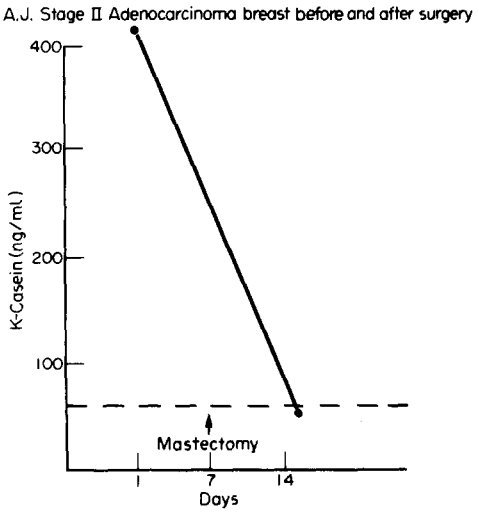
Fig. 1. Distribution of K-casein concentrations in normal subjects, patients with benign breast diseases and patients with breast cancer.

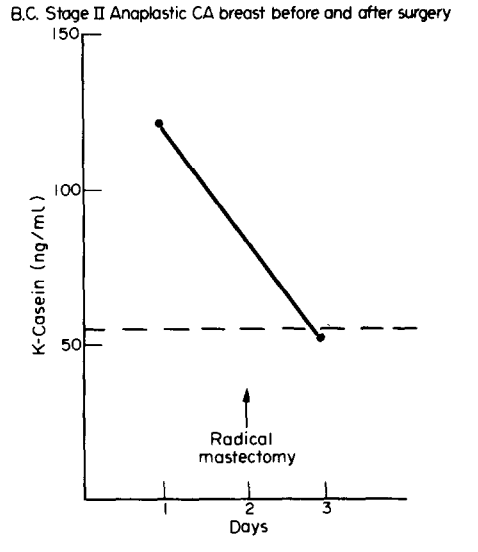
tumours localized to the breast or ipsilateral axillary nodes were assayed. Twenty-one of these (39.6%) had concentrations of K-casein greater than 60 ng/ml whereas 49 of 88 (55.6%) samples from patients with metastatic breast cancer gave results greater than 60 ng/ml (Fig. 1).

No abnormalities were found in glucose, adrenocorticotrophic hormone, growth hormone, cortisol, thyroid-stimulating hormone or prolactin values of six patients with elevated casein values before and after stimulation with insulin, thyrotrophin-releasing hormone, and luteinizing-hormone releasing-hormone. Casein concentrations did not alter following pituitary stimulation.

There was no correlation between casein concentration and age, menstrual status, parity, family history of breast cancer, number of breast-fed children, history of hormone administration, or, in advanced disease, site of metastases. Studies of a possible correlation between K-casein concentrations and the histological type of tumour are in progress.

Serial measurements of K-casein concentrations were performed in a small number of patients (Figs. 2–5). In two patients, K-casein values fell following mastectomy (Figs. 2 and 3). Clinical remissions and relapses correlated with falls and elevations of casein concentration (Figs. 4 and 5), but it is not yet clear whether changes in casein concentrations are sufficiently sensitive to give advance warning of changes in the clinical condition.





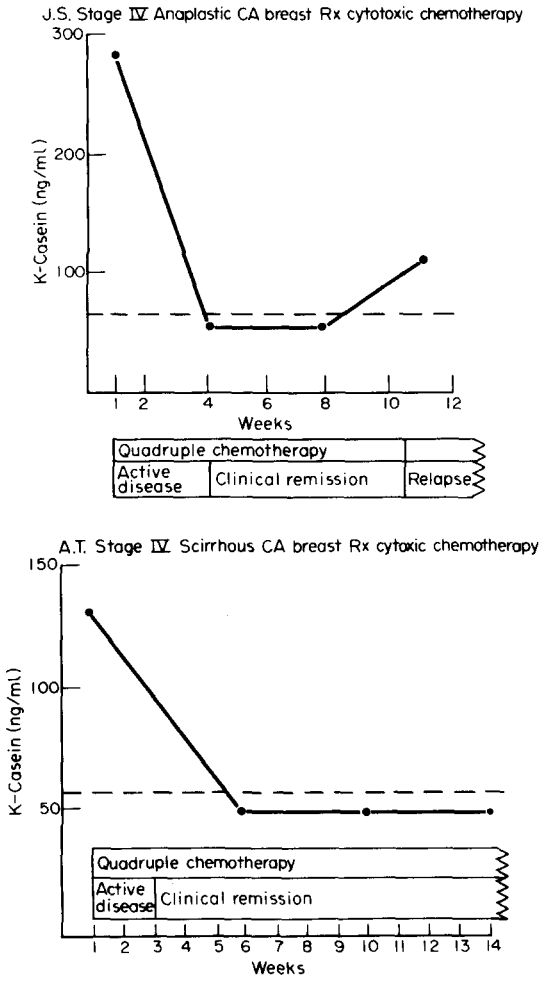
Figs. 2 and 3. K-casein concentrations in two patients before and after mastectomy.

DISCUSSION

Apart from any information which studies of tumour products may yield about the cell kinetics of various cancers, they may be of value in their clinical management. Tumour products already aid screening, diagnosis, treatment monitoring, follow-up and prognosis of various malignant diseases.

Breast cancer is the commonest tumour of women. Current clinical problems include early detection, adequacy of initial therapy, detection and treatment of occult metastatic disease, and treatment of overt metastatic disease. A suitable tumour marker might help solve these difficulties.

The ideal tumour index substance would have several properties: it should be soluble, detectable in peripheral blood, and preferably have a short half-life so that changes in plasma con-



Figs. 4 and 5. K-casein concentrations in 2 patients receiving cytotoxic chemotherapy, showing correlations between these concentrations and clinical conditions.

centrations would accurately and quickly reflect growth or regression of the tumour producing it. K-casein fulfils these criteria although clearly it is not detectable in the serum of all patients with breast cancer and is not unique to breast cancer. Studies to determine the exact half-life of K-casein are in progress but changes in casein concentrations have been noted within 48 hr of removal of tumours by mastectomy.

Many tumour markers are hormones and suffer from the disadvantage of requiring differentiation from the normal hormone concentrations. We have found elevated circulating casein concentrations to be common in benign and malignant breast diseases, other malignancies, and in pregnant and lactating women (Fig. 1), whereas the incidence of positivity in the control samples is low (4.7%) and this degree of specificity may be acceptable for clinical purposes. We have no evidence that casein concentrations are affected by any secondary factors such as physiological variations in the lactogenic hormones.

It seems, however, that any role which K-casein may have as a screening agent for breast cancer may be limited by the relatively high number of positive values in patients with histologically proven benign breast diseases, and by the high number of negative results found in patients subsequently shown to have breast cancer.

Many of tumour-associated antigens are enzymes or hormones found in foetal or placental tissue, and thought to be produced by the tumour as a result of genetic derepression. It seems probable that the mechanism of secretion of this exocrine product may be a similar response to changes in cellular genetic control, induced by the onset of malignancy. The cells most sensitive to such a change would be those already associated with production of the normal product. Thus it may be that mammary gland cells require less "transformation" to produce milk proteins than cells from other organs and this may account for the high incidence in breast cancers. This is similar to the situation with other tumour markers, such as human chorionic gonadotrophin, which is produced by many tumour types, but which is particularly common in the tumours of the placenta and testis.

Immunofluorescent studies are in progress to ascertain whether production of K-casein is directly from the tumour, or whether it is produced by the normal breast tissue as a response to malignancy.

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Relative Importance of Genotype and Type of Mammary Tumor Virus on Mammary Tumorigenesis in Mice*

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Abstract—*Relative importance of genotype and type of mammary tumor virus (MTV) on determining the biological and morphological characteristics of mammary tumors appearing during reproductive states was studied in mice. Females of GR/A§, SHN, their reciprocal F1 hybrids [(GR × SHN)F1 and (SHN × GR)F1, GR/A fosternursed by SHN lactators (GR/SHN) and SHN fosternursed by GR/A lactators (SHN/fGR) were subjected to force breeding until the* 3rd *to the* 5th *pregnancy beginning* 60 days *of age. In GR/A, both F1-hybrids and GR/fSHN, mammary tumor incidence by the* 3rd *pregnancy was* 88–100%, *all tumors were pregnancy-dependent and almost all of them were diagnosed as plaques or mammary tumor type p. On the other hand, in SHN and SHN/fGR, mammary tumor incidence and percentage of pregnancy-dependent tumors were very low and most of the tumors were adenocarcinoma. Numbers of tumors per mouse were* 2.2–3.2 *and about* 1 *in the former four and in the latter two groups, respectively. Taking into consideration of the genotype(s) and type(s) of MTV of each group of mice, these results indicate that the relative importance as factors for determining the characteristics of mammary tumors during the reproductive states in mice is in the order of GR-genotype, SHN-genotype, GR type of MTV and SHN type of MTV.*

INTRODUCTION

GR/A§ MICE [1] is unique in that the growth of mammary tumors is pregnancy-dependent and mammary tumor virus (MTV) is transmitted not only by females but also by males. Genetical control of the transmission of this type of MTV [2–4] and the growth pattern of this type of mammary tumors [5] have been studied. On the other hand, in SHN mice established recently in our laboratory [6], the growth of mammary tumors is autonomous and MTV is transmitted only through milk quite similarly to most high mammary tumor mouse strains [7, 8], although this strain has been established with no relation to the other high mammary tumor strains.

The primary objective of this paper is to evaluate the relative importance of genotype and type of MTV in determining the biological and morphological characteristics of mammary tumors using these two strains with special reference to the tumors which appear during the reproductive states.

MATERIAL AND METHODS

GR/A and SHN strains maintained in the senior author's laboratory were used at F69–70 and F28–29 of brother × sister mating, respectively. Six groups were set up: Group I, GR/A; Group II, SHN; Group III, Fl-hybrid between GR/A females and SHN males [(GR × SHN)Fl]; Group IV, Fl-hybrid between SHN females and GR/A males [(SHN × GR)Fl]; Group V, GR/A fosternursed by SHN [GR/fSHN]; Group VI, SHN fosternursed by GR/A [SHN/fGR]. In Groups V and VI, new born females were removed from their own mothers within 8 hr, mostly immediately after birth, and groups of 6–7 were fosternursed by females of another strain that had began to lactate 2–4 days earlier. At about 60 days of age, every female in Groups I–IV was mated with

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§The name was recently changed to GRS/A (J. Staats, Standard nomenclature for inbred strains of mice: Sixth listing. *Cancer Res.* **36,** 4333, 1976).

one male of corresponding genotype; each female in Groups V and VI was mated with an SHN male. Three to five consecutive pregnancies without subsequent lactation were obtained by removing the young soon after parturition (force-breeding). Each mouse was checked for palpable mammary tumors every day for a few days before and after parturition and every 2 or 3 days during the other period. Number of tumors and tumor size expressed in terms of the geometric mean of the major 2 diameters were recorded. On the day of parturition, some mice in each group were killed by decapitation and mammary tumors were removed and prepared for histologic observation. Pieces of tumors were fixed in Bouin's solution, embedded in paraffin, cut at 6 μm and stained with hematoxylin-cosin. Histologic type of tumors was classified according to the following criteria:

Adenocarcinoma;

Type A = Mammary tumors of basic acinar structure, which are composed mostly of microtubular adenocarcinoma.

Type B = Mammary tumors of basic papillary structure, which are composed of papillary cystadenocarcinoma and pierced by capillaries and blood extravasations.

Type AB = Mammary tumors mixed with Types A and B.

Type C = Fibroadenomatous or adenofibromatous tumors.

Adenoacantoma = Squamous or keratinizing mammary tumors.

Pale-cell or large-cell carcinoma = Solid masses of the large pale cells with enlarged and vesicular nuclei, which reveal more or less cellular atypia [9].

Special forms;

Mammary tumor type p = Mammary tumor is composed of imperfect plaques and small atypical tubuloalveolar or papillomatous hyperplasia, the cells of which are larger than those of the surrounding normal mammary tissue. Some tumors of type p lack organoid structure of plaques but are usually bound together by varying amounts of connective tissue. Type p is usually more clearly demarcated than adenocarcinoma, but the proliferating elements radiate into the tissue of the fat pad [10].

Plaque = Plaque is characterized by abundant overgrowth of tubules. In perfect plaques these systems of branching tubules are symmetrically arranged and form an organoid structure with a loose medulla and a more compact cortex of radially disposed branching tubules. Many variants of this simple arrangement are present (multilobular, giant, irregular comple, etc.) [11].

Throughout the experiment, each couple of mice was kept in a teflon cage (15 × 30 × 12 cm) with wood shavings, maintained in an animal room air-conditioned (24 ± 0.5°C and 65–70% in relative humidity) and artificially illuminated (14 hr light from 5:00 A.M. to 7:00 P.M.) and provided with a commercial diet (CA-1: CLEA Japan Inc. Tokyo, Japan) and tap water *ad libitum*.

RESULTS

Genotype, type of MTV carried and results of mammary tumorigenesis at the 3rd pregnancy in each group are illustrated in Table 1. Most mice had mammary tumors by the 3rd pregnancy (4 months of age) in Groups I, III, IV and V. Meanwhile, only 20 and 35% of mice had tumors in Groups II and VI, respectively. Furthermore, growth of almost all tumors was pregnancy-dependent in the former four groups, but none and only 20% of tumors were pregnancy-dependent in Groups II and VI, respectively. Number of tumors per tumor-bearing mouse in Group II or VI was about half or less than half of those of the other groups. The differences in all parameters were statistically significant between Groups II and VI and the other groups ($P < 0.05$ or 0.01).

Growth curves of mammary tumors in each group expressed in terms of the percentage of the size against that on the day of parturition are presented in Fig. 1. In Groups I, III, IV and V, mammary tumors increased in size during the latter half of pregnancy, peaked approximately on the day of parturition and regressed thereafter. No difference was observed in the pattern of growth between the 2nd and the 3rd pregnancies. On the other hand, mammary tumors in Group II continued to grow after appearance without being influenced by pregnancy. While the growths of 8 out of 10 tumors in Group VI were slightly influenced by pregnancy and parturition, 2 tumors regressed temporarily after parturition. Thus, the average size of tumors in this group showed a temporary reduction after parturition followed by regrowth. However, the pattern of growth curve of tumors in Group VI was rather like that in Group II than in the other groups. These findings in the pattern of mammary tumor growth reflected well with the

Table 1. *Genotype, type of MTV and mammary tumorigenesis in each group*

Group*	Genotype	Type of MTV	Mammary tumorigenesis at the 3rd pregnancy†		
			Mammary tumor incidence (%)	Percentage of pregnancy-dependent tumors (%)	Number of tumors per tumor-bearing mouse
I. GR/A	GR	GR′‡, GR	100 (30/30)§	100 (67/67) ‖	2.3±0.2¶(29)**
II. SHN	SHN	SHN	20 (10/50)	0 (0/12)	1.1±0.2 (10)
III. (GR×SHN)F1	GR, SHN	GR′, GR	92 (110/119)	100 (229/229)	2.5±0.2 (83)
IV. (SHN×GR)F1	GR, SHN	GR′, SHN	100 (97/97)	99 (213/215)	3.2±0.2 (80)
V. GR/fSHN	GR	GR′, SHN	88 (15/17)	100 (33/33)	2.2±0.3 (15)
VI. SHN/fGR	SHN	GR	35 (9/26)	20 (2/10)	1.2±0.1 (9)

*Each group of mice was subjected to force-breeding beginning about 60 days of age until the 3rd to the 5th pregnancy. Females of Groups I–IV were mated with males of the same groups, respectively, and females in Groups V and VI were mated with SHN males.

Groups II and VI were significantly below the other groups in all parameters ($P<0.05$ or 0.01), while there was little difference between Groups II and VI and between Groups I, III, IV and V, respectively.

‡From males and/or females through germinal cells.

§Number of mice with mammary tumors/total number of mice examined.

‖Number of pregnancy-dependent tumors/total number of tumors.

¶Means ± S.E.M.

**Number of mice examined.

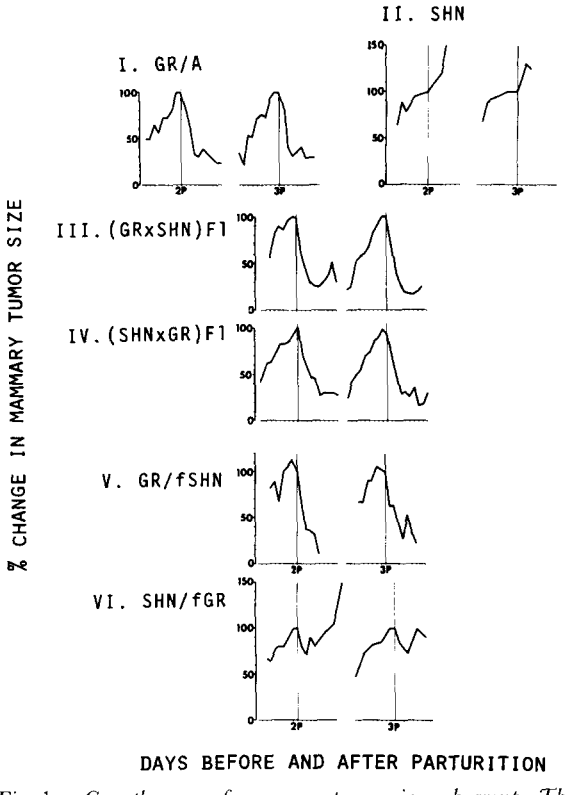

Fig. 1. *Growth curve of mammary tumors in each group. The ordinate is the percentage of mammary tumor size at each stage against the size on the day of parturition. 2P and 3P are the 2nd and the 3rd parturitions, respectively. Each point represents the mean of* 9–35 *estimates.*

percentage of pregnancy-dependent mammary tumors in each group (Table 1).

The results of the histologic type of mammary tumors in each group are illustrated in Table 2. As observed in the biological characteristics, there was similarity in the histologic type of tumors between Groups I, III, IV and V and between Groups II and VI, respectively. More than 74% of tumors belonged to mammary tumor type p and plaques in Groups I, III, IV and V. On the other hand, 100 and 90% of tumors examined were diagnosed as adenocarcinoma type A, B or AB in Groups II and VI, respectively.

DISCUSSION

This study shows that (GR × SHN)F1, (SHN × GR)F1 and GR/fSHN are quite similar to GR/A in the pattern of mammary tumor growth, mammary tumor incidence and number of tumors per mouse. Moreover, more than 74% of tumors were diagnosed as tumor type p and plaques and the percentage of adenocarcinoma were less than 13% in these groups. In this respect, van Nie [10] described that less than half of the pregnancy responsive mammary tumors arose as plaques, the remainder arising as aggregated foci of adenosis and epitheliosis, and that the pregnancy unresponsive tumors had the structure of adenocarcinoma types A and B, the latter being more frequent. Van Ebbenhorst Tengbergen [12] also reported that genetical factors exert an influence on mammary tumor types. GR/A, both F1-hybrids and GR/fSHN have both GR-genotype and GR type of MTV and some groups further contain SHN-genotype and/or SHN type of MTV. Thus, the results infer that both GR-genotype and GR type of MTV would play the limiting roles in determin-

Table 2. Percentage of various types of mammary tumors in each group

Group	No. of tumors examined	Types of mammary tumors (%)*						
		A	B	AB	C	AD	PL	p
I. GR/A	39		10.3		2.5	2.5	10.3	74.4
II. SHN	33	15.1	51.6	33.3				
III. (GR × SHN)Fl	26		7.7	7.7				84.6
IV. (SHN × GR)Fl	34		2.9		2.9	5.9	8.8	79.5
V. GR/fSHN	31		6.4				9.7	83.9
VI. SHN/fGR	37	16.2	54.1	18.9			8.1	2.7

*A: adenocarcinoma type A, B: adenocarcinoma type B, AB: mixed type of A and B
C: adenocarcinoma type C, AD: adenoacantoma, PL: pale cell carcinoma
p: mammary tumor type p and plaques.

ing the biological and morphological characteristics of mammary tumors in mice. However, the superiority of SHN-genotype to GR type of MTV in this process has been proved by the subsequent results that several characteristics of SHN/fGR with SHN-genotype were mostly the same as those of SHN, despite its carrying GR type of MTV. While (GR × SHN)Fl and (SHN × GR)Fl females are heterozygous for genotypes of GR/A and SHN, both behaved as GR/A in all characteristics examined. Finally, it may be concluded from the present results that the relative importance of factors determining the characteristics of mammary tumors which appear during the reproductive states is in the order of GR-genotype, SHN-genotype, GR type of MTV and SHN type of MTV.

It seems that GR type of MTV induces some degree of hormone-dependency on mammary tumors of SHN/fGR in view of the temporary reduction in size of some tumors after parturition in this group, which was not observed in SHN.

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Schedule Dependent Effectiveness of CCNU and 5-Fluorouracil in Experimental Chemotherapy*

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Abstract—*The effect of combination chemotherapy with CCNU and 5-FU given either simultaneously or with a time interval of* 24 hr *was studied in the Lewis lung carcinoma and in the mouse osteosarcoma C22LR. Tumour growth delay, number of lung metastases and lung colonies as well as the survival time of mice with and without surgical removal of the primary tumour were used as parameters. Treatment with CCNU followed by 5-FU was the least effective schedule in both tumour lines.*

In order to evaluate therapeutic gain, we also investigated the cell killing effect of both drugs in different schedules on resting and on rapidly proliferating bone marrow stem cells: CCNU followed by 5-FU was the most toxic treatment sequence. Consequently, the therapeutic effect (the ratio between effect on tumour and effect on normal tissues) must be low when this sequence is given.

If a combination of CCNU and 5-FU is considered for the clinical treatment of gastrointestinal cancer, the most frequently used scheme is a nitrosourea on the first day of a 5-*day course of* 5-*FU. Based on our preclinical studies, we suggest a schedule in which* 5-*FU is followed within a short time interval by CCNU.*

INTRODUCTION

THE MODE of treatment of malignant diseases with cytostatic drugs is strongly influenced by different theories and concepts. Originally, single drugs were given preferably daily, and in low dosages. Later, sequential courses with a different agent were alternated at intervals of a few weeks.

Drugs were increasingly used in combination and, instead of a daily single drug treatment, high dose-intermittent polychemotherapy schedules were gradually applied: many drugs were given together on the same day, and the scheme was usually repeated every 3 or 4 weeks, depending on the bone marrow recovery.

The use of cytostatic drugs in sequences with short time intervals of hours instead of days or weeks is based on various assumptions. The design of treatment schedules is influenced by data from cell kinetics, pharmacokinetics and enzyme kinetics. Tumour cell synchronization in order to increase the cell killing effect of a second drug was recently reviewed [1–3]. The enhancement of the cellular transport of methotrexate by pretreatment with vincristine is an example of sequential treatment based on pharmacokinetic data [4]. The concepts of concurrent and sequential enzyme inhibition of metabolic pathways have a great impact on the drug schedule design [5]. The use of leucovorin after methotrexate treatment is another example of sequential treatment [6]. This short interval sequential treatment is the subject of our study and the selection of the drug combination was based on knowledge of cellular kinetics.

The growth of solid tumours is characterized by a decreasing growth fraction. This will result in a less effective treatment with cell cycle specific drugs such as cytosine arabinoside, methotrexate and 5-FU. Recruitment of cells after tumour cell kill by cytostatic treatment may increase the sensitivity of the tumour to cell cycle specific drugs [7]. Based on this concept, Schabel suggested a sequential treatment with cell cycle non-specific drugs such as CCNU followed by cell cycle specific drugs [8].

We investigated the effect of the sequential

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treatment with CCNU and 5-FU on two different solid tumour lines and, in order to evaluate therapeutic gain, also on critical normal tissue.

The effect of combination chemotherapy with CCNU and 5-FU given either simultaneously or with a time interval of 24 hr on the Lewis lung carcinoma, the mouse osteosarcoma C22LR, and on haemopoietic stem cells, was therefore studied.

MATERIAL AND METHODS

Tumours

The Lewis lung carcinoma, a transplantable poorly differentiated epidermoid carcinoma, arose spontaneously in the lung of a C57BL mouse in 1951 [9]. Our tumour line was obtained from the "Mario Negri" Institute, Milan, Italy, in 1973. Since then, it has been passaged in C57BL/Ka/Rij male mice by bi-weekly s.c. injection of an inoculum of about 10^6 viable tumour cells into both flanks. From a single passage, multiple ampoules of tumour cells were stored in liquid nitrogen. Not more than 8 passages from each ampoule were derived for use in experiments. For the experimental work, the (C57BL/Rij × CBA/Rij)F_1 hybrid male mouse (BCBA) was used. Lewis lung cells, 10^6 cells per 0.1 ml Hanks' balanced salt solution, were injected s.c. into the flank. In order to increase the number of spontaneous lung metastases, Lewis lung cells were injected into the gastrocnemius muscle or into the foot pad of the right hind leg of the recipient mouse. The length and width of tumours in mm were obtained by caliper measurements and converted to volume using the formula: $V = (a \times b^2)/2$ where $b < a$. Volume measurements between 200–800 mm^3 of s.c. and i.m. inoculated Lewis lung cells resulted in a doubling time of 2.5 days. For the assessment of the effect of treatment on artificial lung colonies produced by i.v. injected Lewis lung tumour cells, lungs were removed about 2 weeks after injection and fixed in Bouin's fluid. The lobes were separated and the total number of macroscopic tumour nodules on the lung surface was counted using a dissecting microscope. The effect of treatment on spontaneous lung metastases was assessed by counting the number of lung metastases after a specified time period. After amputation of the leg with the tumour in the foot pad, the mice die of lung metastases. The survival time of mice after post-operative chemotherapy therefore correlates with the effect of treatment on lung metastases.

The strontium-90 induced C22LR mouse osteosarcoma originated in our institute in a mouse in 1957 and was serially transplanted in similar mice or stored in liquid nitrogen [10]. All studies were performed with tumour of passages 78–84 from frozen samples of passage 77. Osteosarcoma cells, 10^6 cells per 0.1 ml Hanks' balanced salt solution, were injected bilaterally s.c. into BCBA male mice and the volume in mm^3 was obtained by caliper measurements. The doubling time, measured between 200–800 mm^3, is 2.5 days. In both solid tumour lines, volume measurements were done at least twice a week. The median tumour volume in mm^3 was calculated from data of at least 4 tumours per treatment group. For the assessment of the effect of treatment on s.c. or i.m. inoculated tumours, groups of 5 mice were selected randomly from a large group of tumour bearing mice.

Preparation of tumour cell separations

The s.c. tumours were excised under aseptic conditions and minced with scissors. A suspension of single cells was prepared by a combined mechanical and enzymatic technique [11]. Fragments of tumour were trypsinized at room temperature in 150 ml Hanks' balanced salt solution containing 0.05% trypsin and a small quantity (~5 mg) of DNase. Mechanical dispersion was accomplished by a rotating razor blade for 10 min. Cells were filtered through 6 layers of nylon gauze under gravity and trypsin activity was halted by cooling and the addition of foetal calf serum. After centrifugation at 4°C and 500 ***g*** for 10 mins, the pellet was resuspended and diluted in Hanks' balanced salt solution with 5% (v/v) foetal calf serum. Viability was checked by eosine staining. It usually exceeded 75%.

Haemopoietic stem cell systems

For the determinations of the activity of drugs against normal cells, we used the haemopoietic stem cells from the mouse, since bone marrow is often the tissue that defines the tolerance to cytostatic treatment. The survival of haemopoietic stem cells was determined by the spleen colony technique of Till and McCulloch [12]. Groups of 5 BCBA male mice were treated by i.p. injection of drugs. CCNU was given in a dose of 15 mg/kg and 5-FU in a dose of 50 mg/kg. Sixteen hours after the last treatment, the bone marrow was flushed from the femurs with cold Hanks' balanced salt solution. After filtration through nylon gauze, suitable dilutions of the pooled femurs marrow were injected i.v. into groups of 10 lethally irradiated isogenic male mice (950 rad of gamma rays from a 611 Ci ^{137}Cs source at an exposure rate of 122 rad/min 1–2 hr earlier). Nine days later, the spleens of these

recipients were fixed in Tellyesniczky solution. The spleen colonies with a dia of 0.25 mm or more were counted under a dissecting microscope. The results were expressed as the number of colony forming units (CFU) per donor femur. These results were compared with the results of a simultaneous assay in a group of untreated donor mice to determine the relative CFU survival.

After treatment with a cytostatic drug, the normally resting haemopoietic stem cells will be recruited into cycle and this may result in increased drug sensitivity. Therefore, we also looked into the effect of treatment on recruited, rapidly proliferating normal bone marrow stem cells. Lethally irradiated male mice (950 rad) received i.v. injections of a standard amount of bone marrow (one quarter of a femur content). Six days later, their spleens contained a large number of colony forming stem cells in a phase of rapid proliferation [13]. CCNU was given in a dose of 20 mg/kg and 5-FU in a dose of 25 mg/kg. Drugs were administered i.p. to groups of 5 mice.

Sixteen hours after the last treatment spleen cell suspensions were prepared and assayed for the number of CFU as described above. The survival of rapidly proliferating CFU was calculated at the ratio of the mean number of CFU per spleen of treated to that of simultaneously assayed control mice.

Chemicals

CCNU, NSC-79037; CAS reg. No. 13010–47–4; 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea was kindly supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. The drug was injected i.p. as a finely divided suspension in 2% carboxymethylcellulose; the mixture was homogenized before being injected. The particles were fine enough to pass easily through a 25 gauge needle. 5-Fluorouracil, NSC-19893; CAS reg. No. 51–21–8; 5-FU was kindly supplied by Hoffman La Roche, Basel, Switzerland.

Treatment schedule

The drug sequences we tested were 5-FU and CCNU given simultaneously, 5-FU was given 24 hr before (5-FU→CCNU) or 24 hr after CCNU (CCNU→5-FU), Since we kept this interval constant in all experiments, no information was obtained on a time response relationship. For each solid tumour experiment, the day of CCNU treatment was kept constant; the day of 5-FU treatment was varied. In the stem cell experiments, the time of the last drug treatment and of assay were kept constant.

Endpoints and statistical evaluation

The end-point of treatment of both solid tumour lines is the growth delay in days; the displacement in time between the growth curve of the tumours in the control group and the growth curve of the tumours recurring after treatment. The displacement between the growth curves is chosen at a point, usually close to the original tumour volume, where the growth curves for treated and control animals are again parallel [14]. If no original tumour volume is known (e.g., for tumour inocula exposed to treatment before a tumour is palpable), the delay in comparison with the control group to reach an arbitrary volume between 400–800 mm^3 is estimated. The endpoint of the lung assay is the number of artificial lung colonies or number of spontaneous lung metastases per mouse. Survival experiment results are expressed in median survival time in days. The end-point of the effect of treatment of resting and rapidly proliferating haemopoietic stem cells is the surviving cell fraction. The Student's *t*-test is used for the evaluation of the results of treatment with the different sequential combinations.

RESULTS

Table 1 shows the effects for the combination CCNU and 5-FU on Lewis lung carcinoma if administered to the mice before tumours are palpable; 5-FU given 24 hr before or simultaneously with CCNU seems to enhance the effect of CCNU. The sequence CCNU→5-FU is the least effective. The effects of the single agents and their combinations in the treatment of palpable tumours using both tumour volume and the number of spontaneous lung metastases were then investigated. As shown in Table 2, there is no effect of CCNU or 5-FU alone on tumour volume, but the number of visible spontaneous lung metastases in these groups is very low in comparison with the control group, Note that the average number of lung metastases in the fourth group is unexpectedly high. To increase the number of spontaneous lung metastases, Lewis lung tumour cells were inoculated i.m. instead of s.c. in the subsequent experiments. In Table 3, the influence of the sequence of drug treatment on primary tumour growth and lung metastases is clearly seen. The effect of treatment on the number of artificially induced lung colonies is shown in Table 4. A similar pattern emerges as is seen in Tables 1, 2 and 3. The effect of treatment on mouse survival time was followed in order to see whether this was

Table 1. The effect of sequential treatment on tumour volume in Lewis lung carcinoma

Treatment schedule	Tumour volume day 13 (mm^3) Median (range)	Mice with tumour on day 13/ total number of mice
Control	900 (1624–720)	5/5
5-FU	637 (1062–450)	5/5
CCNU	320 (598–100)	5/5
CCNU→5-FU	90 (315– 12)	5/5
CCNU+5-FU	0 (315– 12)	0/5
5-FU→CCNU	0	0/5

Groups of 5 C57BL/Ka male mice (20 g) were inoculated s.c. with 10^6 Lewis lung tumour cells on day 0. CCNU 20 mg/kg i.p. was given on day 3. A dose of 100 mg/kg 5-FU i.p. was given either simultaneously, 24 hr before or 24 hr after CCNU.

Table 2. The effect of sequential treatment on tumour volume and on spontaneous lung metastases in Lewis lung carcinoma

Treatment schedule*	Tumour volume day 21 (mm^3) Median (range)	Growth delay (days) Mean ± S.E.	Number of lung metastases per mouse Mean ± S.E.
Control	2737 (3496–1012)	0 ± 0.5	16.2 ± 3.9
CCNU	2352 2572–1450)	0 ± 0.5	1.2 ± 0.4
5-FU	2047 (2562–1237)	1.0 ± 0.3	3.2 ± 1.1
CCNU→5-FU	800 (1045– 504)	7.0 ± 0.6	8.7 ± 3.2
CCNU+5-FU	105 (504– 82)	12.5 ± 1.5	0
5-FU→CCNU	605 (182–1326)	12.0 ± 3.0	0

*As in Table 1; CCNU was given on day 11 when the median tumour volume was 450 mm^3. Growth delay was calculated at a tumour volume of 600 mm^3. Lung metastases were counted on day 30.

Table 3. The effect of sequential treatment with different drug dosages on Lewis lung carcinoma expressed as growth delay and number of lung metastases per mouse

Treatment schedule	5-FU and CCNU dosage in mg/kg i.p.					
	100 and 20		100 and 10		50 and 20	
Control	0 ± 0.5*	76.4 ± 3.2†	0 ± 0.5*	76.4 ± 3.2†	0 ± 0.5*	76.4 ± 3.2†
5-FU	5.5 ± 0.4	43.2 ± 4.1	5.5 ± 0.6	43.2 ± 4.1	4.0 ± 0.4	49.0 ± 1.0
CCNU	7.5 ± 1.0	23.0 ± 3.6	3.5 ± 1.0	50.0 ± 4.1	7.5 ± 1.0	23.0 ± 3.6
CCNU→5-FU	a 15.5 ± 1.0	b 13.0 ± 2.6	c 6.5 ± 0.5	d 56.7 ± 2.4	a 9.0 ± 1.0	e 18.8 ± 2.1
CCNU+5-FU	a 17.0 ± 1.0	b 4.2 ± 2.6	c 7.0 ± 1.5	d 19.5 ± 3.6	a 4.5 ± 0.5	e 12.8 ± 1.8
5-FU→CCNU	a 23.0 ± 1.5	b 1.0 ± 0.6	c 7.0 ± 1.5	d 20.7 ± 3.7	a 13.5 ± 1.0	e 13.2 ± 2.0

Groups of 5 BCBA male mice (20 g) were inoculated i.m. with 10^6 Lewis lung carcinoma cells. CCNU was given on day 3 and 5-FU was given either simultaneously, 24 hr before or 24 hr after CCNU. Lung metastases were counted on day 32.
*Growth delay in days. Mean with S.E.; (a) $P<0.01$; (c) not significant.
†Number of lung metastases per mouse. Mean with S.E.; (b) $P<0.0025$; (d) $P<0.0005$; (e) $P<0.05$.

similarly affected as was tumour growth delay. Table 5 shows the median life span of mice with Lewis lung tumour. The more than additive effect of the combination and the influence of drug sequence on the median life span are in agreement with the data obtained by volume measurements. Figure 1 shows the results of experiment II in Table 5 in detail: CCNU→5-FU treatment results in a number of early toxic deaths, presumably caused by bone marrow failure. On the other hand, when 5-FU is followed by CCNU, 2 out of 10 animals were without evidence of disease 82 days after inoculation. The effect of adjuvant chemotherapy is shown in Table 6.

In order to investigate whether treatment of another tumour system with CCNU and 5-FU shows similar responses to different sequences, we treated s.c. transplanted osteosarcoma in BCBA mice. Table 7 shows the effects of a single

Table 4. The effect of sequential treatment on number of lung colonies in Lewis lung carcinoma

Treatment schedule	Number of lung colonies per mouse Mean ± S.E.	
Control*	86.7 ± 8.8	
5-FU	92.0 ± 8.0	
CCNU	20.6 ± 6.4	
CCNU → 5-FU	9.8 ± 3.0	$P < 0.01$
CCNU + 5-FU	1.5 ± 0.5	
5-FU → CCNU	1.5 ± 0.5	

One million Lewis lung tumour cells in 0.5 ml Hanks' balanced salt solution were injected into one of the tail veins of C57Bl/Ka male mice. Lung colonies were counted on day 17.

*Two out of 5 animals of the control group died on day 17 as a result of lung disease. Treatment schedule as in Table 1; CCNU was given on day 3.

drug treatment and the influence of the drug sequence: CCNU → 5-FU seems to be the least effective treatment schedule. The effects of sequential treatment on haemopoietic stem cells are shown in Table 8.

DISCUSSION

The results indicate that, for haemopoietic stem cells, either in a resting or in a rapid proliferation state, CCNU → 5-FU gives the lowest survival fraction in comparison with the other treatment schedules. The combination of 5-FU and CCNU shows schedule dependency: CCNU → 5-FU is the most toxic sequence for the normal tissue as represented by bone marrow stem cells. This same sequence, CCNU → 5-FU, however, seems to be the least effective sequence

Table 5. The effect of sequential treatment on life span of mice with Lewis lung carcinoma

Treatment schedule	Median survival time and range of death (days) Experiment I	Experiment II
Control	35 (26–38)	34 (23–42)
5-FU	39 (27–42)	35 (30–41)
CCNU	44 (34–49)	35 (33–50)
CCNU → 5-FU	47 (14–73)	28 (13–44)†
CCNU + 5-FU	52.5 (40–)*	36 (34–48)
5-FU → CCNU	61 (40–)*	45 (36–)*

Groups of 10 BCBA male mice (20 g) were inoculated i.m. with 10^6 Lewis lung tumour cells on day 0. CCNU 20 mg/kg i.p. was given on day 4 in experiment I and on day 6 in experiment II. 5-FU in a dosage of 100 mg/kg i.p. in experiment I and of 50 mg/kg i.p. in experiment II was given simultaneously, 24 hr before or 24 hr after CCNU.

*Two out of ten mice had no apparent tumour growth at the implantation site at the end of the experiments (82 days).

†The wide ranges were due to early toxic deaths.

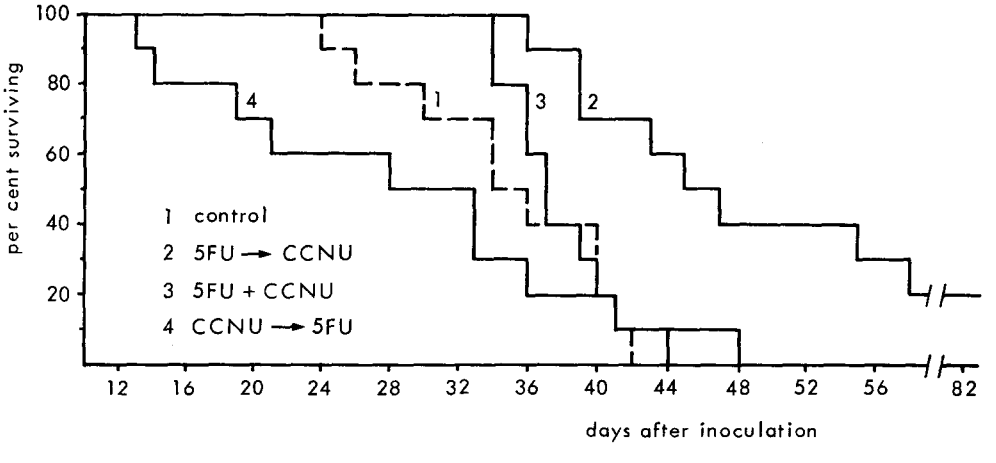

Fig. 1. Groups of ten BCBA mice were inoculated i.m. with one million cells. Six days later treatment was given. Dose: 5-FU, 50 mg/kg i.p. and CCNU, 20 mg/kg i.p.

Table 6. The effect of adjuvant chemotherapy with different drug sequences on the life span of mice with Lewis lung carcinoma

Treatment schedule	Median survival time and range of death (days) Experiment I	Experiment II
Control	44 (21–69)	71 (33–88)*
5-FU	54 (33–57)	71 (34–82)
CCNU	58 (33–59)	74 (44–88)
CCNU→5-FU	53 (45–59)	75 (37–86)
CCNU+5-FU	59 (47–69)	75 (32–90)
5-FU→CCNU	88 (59–)*	82 (32–90)

In experiment I. BCBA male mice were inoculated into the foot pad with 5×10^5 Lewis lung tumour cells in 0.02 ml Hanks' balanced salt solution on day 0. Tumour volume at the time of amputation on day 18 was approximately 500 mm^3. On the seventh post-operative day, groups of 8 mice were treated with CCNU 10 mg/kg i.p. A dose of 100 mg/kg 5-FU i.p. was given either simultaneously, 24 hr before or 24 hr after CCNU. Animals died of lung metastases. In experiment II, C57BL/ka male mice were used; the treatment procedure was as in experiment I.

*Four out of 8 animals had no evidence of disease on day 120.

†The last 2 mice in each treatment group were sacrificed for macroscopic lung investigation: all animals showed multiple, mostly large, lung metastases.

Table 7. The effect of sequential treatment in osteosarcoma

Treatment schedule	Mean growth delay and standard errors (days) Experiment I		Experiment II	
Control	0±0.3		0±0.3	
5-FU	4.5±0.3		3.3±0.1	
CCNU	9.0±0.7		3.5±0.1	
CCNU→5-FU	12.0±0.8	$P<0.025$	5.0±0.2	$P<0.0005$
CCNU+5-FU	13.5±1.2		7.5±0.4	
5-FU→CCNU	17.0±0.7		7.0±0.2	

One million osteosarcoma cells were injected bilaterally s.c. into groups of 5 BCBA male mice (23 g) on day 0. CCNU 20 mg/kg i.p. was given on day 3. A dose of 100 mg/kg i.p. 5-FU was given either simultaneously, 24 hr before or 24 hr after CCNU.

Table 8. Percentage of surviving cells per femur and repopulating spleen in comparison with untreated control mice

Treatment schedule	Resting stem cells Exp. I	Exp. II	Proliferating stem cells Exp. I	Exp. II	Exp. III
CCNU→5-FU	1.69	7.83	0.70	2.38	0.21
CCNU+5-FU	13.50	13.30	2.33	3.46	5.78
5-FU→CCNU	17.50	25.82	4.13	10.91	9.48

in both solid tumour lines investigated. Consequently, the therapeutic effect (the ratio between the effect on the tumour and the effect on normal tissues) must be low when this sequence is given. Based on the concept of growth fraction, recruitment and increased sensitivity to cell cycle specific drugs, Schabel suggested sequential treatment for solid tumours with first an alkylating agent (in our experiments CCNU) followed by an anti-metabolite (in our experiments 5-FU). The data shows that CCNU →5-FU is most effective against the bone marrow, but least effective on the 2 malignant solid tumour lines, making this sequence pro-

bably unsuitable for clinical practice. We would like to suggest that the optimal treatment schedule is 5-FU→CCNU. In our assay system, this sequence is least toxic for haemopoietic stem cells and the results with the 2 solid tumour lines indicate that 5-FU→CCNU may be preferable to simultaneous administration.

Why is CCNU→5-FU the most toxic sequence for bone marrow? CCNU, an alkylating agent, kills resting and proliferating tumour cells with equal effectiveness [15]. After CCNU treatment, recruitment will take place in the resting stem cell model and, because 5-FU is more effective on proliferating than on resting cells [16], an enhanced effect of 5-FU may be anticipated. However, increased sensitivity to 5-FU due to recruitment cannot play an important role in the maximally recruited proliferating stem cell situation [13].

Why is CCNU→5-FU the least effective sequence for the two solid tumour lines? Earlier, we showed the competition between a nucleoside analogue and different nucleosides at the uptake level of tumour cells [17]. As a result of effective treatment, cells will lyse and nucleosides and possibly nucleotides may be released. The concentration of these metabolites, especially with solid tumours, may become high enough to inhibit the effect of a nucleoside analogue such as 5-FU. This could explain the relative ineffectiveness of CCNU→5-FU treatment of Lewis lung carcinoma and osteosarcoma. This hypothesis is tentative in character; a conclusive explanation for the different schedule dependency of 5-FU and CCNU in bone marrow, on the one hand, and the 2 solid tumour lines, on the other, cannot be made.

Is there a synergism between 5-FU and CCNU? The combination of Me-CCNU and 5-FU has been claimed to be modestly potentiating against the experimental colon carcinoma No. 38 and the P388 leukemia [18]. Similarly, a synergistic effect on AKR lymphoma with 5-FU administered 4 hr before BCNU has been described [19]. Our results of treatment of Lewis lung carcinoma suggest a more than additive effect for a particular drug sequence. Since the dose–effect curve for growth delay is usually not linear, the identification of synergism is uncertain. For this reason, we have mainly emphasized the differences in effect seen when sequences are varied, without drawing conclusions on synergism.

What may be the clinical relevance of our study? We tested different drug sequences on resting and recruited bone marrow cells and found the sequence CCNU→5-FU to be the most toxic. In two different solid tumour lines as well as in one of our experimental colon tumour lines [20], the same sequence was shown to be least effective. The ultimate proof of which therapeutic regimen is the best can only be obtained by doing a clinical trial; nevertheless, preclinical research may give leads to which treatment is worthwhile testing. The extrapolation of data on model tumours to clinical treatment is uncertain; however, there is more agreement on effects on normal tissues. Since, in this respect, the sequence under discussion also points to the disadvantage of giving 5-FU after CCNU, it seems reasonable to test other sequences.

For the treatment of gastrointestinal tumours, one of the nitrosoureas such as CCNU, BCNU or Me-CCNU is usually given on the first day of a 5-day course of 5-FU with or without a third drug such as vincristine [21–30]. Drug dosages and schedules as well as the length of the 5-FU course are based on the results of Phase-II studies and most probably based on historical grounds as well. From our broad spectrum analysis, we would like to suggest a treatment schedule starting with a short course on 5-FU followed by CCNU.

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Two Methods for Measurement of Oestradiol-17β and Progesterone Receptors in Human Breast Cancer and Correlation with Response to Treatment*

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Abstract—*Two simple quantitative methods, one employing a dextran-charcoal (DCC) procedure, the other using gel filtration on Sephadex LH-20 for separation of bound from free radioactive steroid, have been developed for measurement of specific oestradiol-17β receptor (E_2R) and progestin receptor (PgR) activities in the same tumour cytosol. The synthetic progestin ^3H R5020 (Roussel-UCLAF), was used in the PgR assays. Good agreement was observed between the estimations obtained from the methods for either receptor activity. Either method had a sensitivity level of* 5 fmole/mg *cytosol protein for E_2R and* 15 fmole/mg *for PgR. Dissociation constants obtained by Scatchard analyses from DCC and LH-20 assays were respectively* 2.2 ± 0.2 *(S.E.M.)* $\times 10^{-10}$ *M and* $2.6 \pm 0.3 \times 10^{-10}$ *M for E_2R,* $7.7 \pm 0.7 \times 10^{-10}$ *M and* $7.6 \pm 0.7 \times 10^{-10}$ *M for PgR.*

Of 71 *primary and* 43 *metastatic human breast tumours examined* 62 *and* 51% *respectively were found to have E_2R+ activity. Approximately* 63% *of all tumours with E_2R+ activity were also PgR+. In no instance was PgR+ activity found in a tumour which was E_2R-. Of* 30 *patients with advanced metastatic breast cancer,* 62% *of those with E_2R+ tumours had a clinical response to endocrine therapy. Our present data suggest that the presence of both E_2R and PgR in a tumour may represent a much more accurate indication of a tumour's responsiveness to hormone therapy than the presence of E_2R alone.*

No linear correlation existed between concentrations of E_2R and PgR in primary or in metastatic tumours, nor did the PgR concentration (in contrast to that of E_2R) bear any relationship to the patient's age.

INTRODUCTION

THE SUGGESTION was made by Jensen *et al.* [1] that the presence or absence of oestradiol receptor sites in human breast cancer tissue might be used to predict which tumours were likely to respond to endocrine therapy. In approximately one-third of patients with metastatic breast cancer, the tumour responds favourably to either additive or ablative forms of endocrine therapy. It would be especially desirable to select patients for ablative procedures, such as adrenalectomy or hypophysectomy, as accurately as possible, as these patients are often quite ill and should perhaps be spared these procedures if there was little or no likelihood of success.

Pooled data from several centres has indicated that 55–60% of patients with positive oestradiol receptor (E_2R) activity in their tumour show objective remission to some form of endocrine manipulation [2] whereas less than 10% of those lacking E_2R in their tumour show any response. The fact that 40% of E_2R+ tumours fail to respond indicates the necessity for additional marker(s) to identify the responsive tumour.

The first evidence of specific progesterone-binding components in human mammary carcinoma was provided by Terenius [3]. Horwitz *et al.* [4] proposed that since in oestrogen target tissue, synthesis of progesterone receptor (PgR) is dependent upon oestrogen action [5], the

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presence of PgR in addition to E_2R in malignant cells would indicate that the tumour was capable of synthesizing at least one end product under oestrogen regulation and should therefore be expected to remain endocrine responsive.

In this report we describe two simple quantitative methods, one based on the dextran–charcoal procedure of Korenman and Dukes [6] and the other using gel filtration on Sephadex LH-20 as suggested by Ginsburg *et al.* [7] which we have used, with satisfactory agreement between estimations, to determine both E_2R and PgR activity in the same tumour cytosol. Since progesterone, in addition to binding to its own receptor, binds also to glucocorticoid receptors and to corticosteroid binding globulin the use of ^{3}H-progesterone to assess specific PgR presents difficulties. For assay of PgR sites, we have therefore used the synthetic progestin, R5020 (17, 21-dimethyl-19-nor-4, 9-pregnadiene-3, 20-dione), which has been found to bind specifically to PgR in immature rat and mouse uteri [8].

The methods have been employed to assess the relative frequency of occurence of E_2R and PgR in both primary and metastatic tumours. In a small group of patients with advanced metastatic breast cancer, clinical response to hormone therapy has been correlated with receptor activity.

MATERIAL AND METHODS

Material

[2, 4, 6, 7-^{3}H] Oestradiol-17β [^{3}H-E_2] (spec. act. 100 Ci/mmole) was obtained from the Radiochemical Centre, Amersham. Diethylstilboestrol (DES) was purchased from Koch-Light. [6, 7-^{3}H] 17, 21-dimethyl-19-nor-pregna-4, 9-diene-3, 20-dione [^{3}H-R5020] (spec. act. 50 Ci/mmole) and radio-inert R5020 were a gift from Dr. J.-P. Raynaud (Roussel-UCLAF).

Dextran T-70 and Sephadex LH-20 were purchased from Pharmacia Ltd.; gelatin from the Sterling Gelatine Co. Ltd. and Norit-A-charcoal from Sigma Ltd. Scintillator Grade xylene and PCS liquid scintillation cocktail were supplied by Hopkin and Williams, Chadwell Heath, Essex.

All other reagents used were of Analar grade.

Buffer solutions

The following buffers were used as indicated:

Buffer A for oestradiol receptor (E_2R) measurement: 10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA, 3 mM sodium azide and 0.5 mM dithiothreitol.

Buffer B homogenisation medium for preparation of cytosols for measurement of both E_2R and progesterone receptors (PgR): 10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA, 12 mM thioglycerol, 3 mM sodium azide and 30% (v/v) glycerol.

Buffer C for PgR measurement: as buffer B with 10% (v/v) glycerol in place of 30%.

Steroid solutions

The labelled steroids were stored in convenient sealed aliquots (100 μl) at -20°C as received from the manufacturers: ^{3}H-E_2 in benzene–ethanol 95:5; ^{3}H-R5020 in toluene–methanol 9:1.

Stock solutions. The solvents were evaporated off from an aliquot under a stream of nitrogen and the residue dissolved in ethanol and stored at -20°C in sealed 200 μl aliquots at concentrations of 100 pmole/ml for ^{3}H-E_2 and 200 pmole/ml for ^{3}H-R5020.

Working solutions. ^{3}H-E_2 stock solution was diluted with buffer A to a concentration of 3 pmole/ml and each day from this working solution there were prepared a range of concentrations of from 1.5 to 0.25 or 0.125 pmole ^{3}H-E_2/ml buffer A. ^{3}H-R5020 stock solution was diluted with buffer C to a concentration of 8 pmole/ml and each day a range of concentrations of from 4.0 to 0.375 pmole ^{3}H-R5020/ml buffer C was prepared.

Non-radioactive compounds. Stock solutions (1 mM) of DES and R5020 in ethanol were stored at 0°C and diluted directly with buffers A and C, respectively, to give working solutions of 2×10^{-7} M DES and 8×10^{-7} M R5020.

Dextran-coated charcoal (DCC) suspension

DCC suspension (0.5% charcoal, 0.05% dextran, 0.1% gelatin) was prepared by dissolving 1 g gelatin in 200 ml 10 mM Tris buffer pH 7.4; 40 ml of this 0.5% gelatin solution were used to dissolve 100 mg dextran T70, 1 g charcoal was added with stirring, the volume made up to 200 ml with Tris buffer and the DCC suspension stirred for 1 hr at 4°C. The ice-cold suspension was stirred continuously during subsequent sampling.

Preparation of cytosol

Samples of primary breast tumour tissue were obtained at the time of mastectomy. Biopsies from metastatic skin deposits were taken under local anaesthetic. The samples were freed from surrounding fat and connective tissue, cut to

convenient size and placed immediately in vials in liquid nitrogen. In our experience tumours stored in liquid nitrogen retain oestradiol receptor activity for more than a year. Five hundred milligrams of tumour tissue was obtained: the assay procedure may be carried out with as little as 100 mg but in such cases the cytosol will have a low protein concentration which may lead to less reliable results.

Homogenization was carried out using a "microdismembrator" (Braun, Melsungen, Germany) as recommended by the E.O.R.T.C. Breast Cancer Cooperative Group [9]. The frozen tissue was placed on the wall of a 7 ml Teflon vial, free of a 10 mm tungsten carbide ball at the bottom. The lid was sealed in position with adhesive tape and the vial placed in a flask of liquid nitrogen for 10 min. The Teflon vial was then fixed in the instrument and vibrated for 30 sec at maximum frequency. The frozen powder was transferred rapidly to a 10 ml glass beaker and dispersed in about 4 vol buffer B by stirring for 10 min at 4°C with a magnetic stirrer. The homogenate was transferred by Pasteur pipette to 2.5 ml cellulose nitrate reduction tubes and centrifuged for 60 min at 100,000 × ***g*** and 4°C. The clear cytosol supernatant was removed with a Pasteur pipette, care being taken to leave behind the upper fatty layer.

Wherever possible, E_2R and PgR assays were carried out immediately after preparation of the cytosol. Whereas buffer A may be used in place of buffer B for preparation and storage of cytosols for E_2R measurement alone, it is preferable to use buffer B where the cytosol is required for both E_2R and PgR assay, since glycerol markedly increases PgR stability. Cytosols with protein concentrations in the range 1.0–2.5 mg/ml were found to give satisfactorily reproducible saturation curves for E_2R and PgR in both assay methods used and to retain activity on storage in liquid nitrogen for at least 3 weeks in respect of PgR and up to 10 weeks for E_2R. It is essential to ensure that the cytosol is kept at 0–4°C at all times during its preparation to preserve the activity of the receptors.

Oestradiol-17β receptor (E_2R) *assay*

One hundred microlitres of cytosol were incubated with the same volume of a range of concentrations of ^{3}H-oestradiol-17β in a final volume of 300 μl for 30 min at 30°C or overnight at 4°C, control experiments having shown that comparable binding equilibrium was attained under either condition. A minimum of 8 concentrations of oestradiol were normally used. A second set of tubes contained a 60 × excess of DES. Diethylstilboestrol was preferred to unlabelled E_2 as competitor for specific receptor sites since it is less susceptible to surface adsorption [7].

The bound and unbound E_2 fractions after incubation of cytosol with E_2 were separated by one of the following techniques:

(a) *Dextran-coated charcoal method.* This procedure was based on the method of Korenman and Dukes [6].

Five hundred microlitres of ice-cold DCC solution was added to each incubation tube. The contents of the tubes were well mixed and the tubes left to stand in ice for 10 min before centrifugation at 2000 × ***g*** for 15 min at 4°C. Unbound ^{3}H-E_2 was removed by the charcoal. Five hundred microlitres of the supernatant fluid, containing the bound ^{3}H-E_2 was used to measure the radioactivity.

A duplicate reagent blank was prepared for each concentration of ^{3}H-E_2 used, in which buffer replaced the cytosol. Reagent blanks were incubated and submitted to the same procedure as tubes containing cytosol. The total amount of radioactivity available at each concentration was determined from a series of tubes prepared as for the reagent blanks except that 500 μl buffer was added in place of DCC suspension.

(b) *Gel filtration on Sephadex LH-20.* The method followed was based on that described by Ginsburg *et al.* [7] for the study of high-affinity oestradiol binding in brain and pituitary. Small glass columns (Pasteur pipettes) internal dia 0.5 mm equipped with glass wool plugs were loaded with sephadex LH-20 pre-equilibrated in buffer A (for E_2R) or C (for PgR), to a bed height of 6 cm. The columns were mounted in sets of 24 in rubber bungs fitted into a perspex stand which was provided with a well underneath each column to hold a scintillation vial. When column effluents were not being collected in vials, as, for example, during regeneration, they were allowed to drip into a common basement tray. Columns were maintained in a chamber at 0–4°C during use to minimize dissociation of high affinity bound complexes. Flow through columns was stopped simply by allowing the eluant level above the column to fall to the surface of the gel, capillary action preventing entry of air into the bed.

After use, the LH-20 was regenerated by washing with ethanol (2 ml) followed by buffer (2 ml) which effectively freed the beds of residual radioactivity. Air bubbles were eliminated from the bed during regeneration. The LH-20 could then be re-used without detectable change in its properties over a period of at least 12 months. When not in use, columns were left with a few cm

of buffer above the surface and sealed with small rubber bungs.

Two hundred microlitre aliquots of incubates were applied to the columns and washed in with 100 μl buffer. Flow was allowed to stop for 30 min in order to permit maximum reduction of low affinity, high capacity non-specific bound steroid by differential dissociation as recommended by Ginsburg *et al.* [7], without appreciably affecting the specific high affinity binding. The receptor bound hormone was eluted directly into a scintillation vial by passage of 700 μl buffer, that volume of buffer previously determined as necessary to ensure elution of the complete bound hormone peak without breakthrough of free steroid.

Progesterone receptor (PgR) *assay*

The procedures followed were the same as those used in the measurement of oestrogen receptor, except that ^{3}H-R5020 and radio-inert R5020 were used in place of ^{3}H-oestradiol-17β and DES; buffer C replaced buffer A. Incubation was carried out for 2.5–3 hr at 4°C or overnight as convenient. Comparative assays with tumour cytosol have shown that binding of ^{3}H-R5020 was essentially complete after 2.5 hr and that the concentration bound remained constant over 18 hr.

Measurement of radioactivity

Five hundred microlitres of supernatant fluid following DCC treatment or 700 μl eluate from the sephadex LH-20 column were dispersed in 10 ml of a 1:1 dilution with Scintillator Grade xylene of PCS–liquid scintillation cocktail and counted for 2 min in a Nuclear Chicago Isocap/300 liquid scintillation system using Programme 1B. Counting efficiency was approximately 38%. In the LH-20 method, total counts available at each concentration of radioactive steroid were determined by incubating 300 μl buffer C with the appropriate concentration of radioactive steroid and adding 200 μl of this incubate with a further 500 μl buffer to the scintillation fluid.

Protein determination

The protein content of the cytosol was determined by the method of Lowry *et al.* [10] with bovine serum albumin as standard. Correction was made for the effect of glycerol on the colour reaction when the cytosol was prepared in buffer B.

Analysis of data

Bound steroid was estimated by subtracting the radioactivity present in those tubes containing unlabelled competitor (non-specific binding) from the total bound radioactivity in the tubes without competitor with correction for reagent blanks. The data were then analysed according to Scatchard [11]. Counts/min were expressed as pmole/steroid bound/ml cytosol and plotted against the ratio of bound to free steroid (B/F). Where a single binding component is present the plot gives a straight line the reciprocal of whose slope represents the dissociation constant, K_d. The intercept with the abscissa represents the number of receptor sites in pmole/ml cytosol.

Results were expressed in terms of fmole/mg cytosol protein and were only considered as indicating the presence of positive receptors where a satisfactory Scatchard straight line plot was obtained with K_d values in the range 0.5–5.0 $\times 10^{-10}$ for E_2R and 2.0–14.0 $\times 10^{-10}$ for PgR.

RESULTS

Comparison of results obtained by DCC and Sephadex LH-20 methods

(a) *Oestradiol-17β receptors* (E_2R). Figure 1(a) shows a representative ^{3}H-E_2 binding curve obtained for typical oestradiol receptor positive (E_2R+) tumour cytosol. The Scatchard plot, Fig. 1(b), of the high affinity binding appeared rectilinear, indicating the presence of a single class of high affinity receptor.

A comparison was made between the estimates of specific oestradiol binding capacity obtained by the two methods for 17 tumour cytosols with E_2R+ values ranging from 5 to 250 fmole/mg cytosol protein. In each case, the estimation by the DCC method was made at the time of preparation of the cytosol whereas the LH-20 assay on 75% of the samples was carried out after the cytosol had been preserved from 1–10 weeks in liquid nitrogen. There was no significant difference between the estimates obtained from the two methods. The correlation coefficient was statistically highly significant: $r = 0.91$, $P \ll 0.001$. The regression line equation was $\log(E_2R - \text{LH-20}) = 1.03 \log(E_2R - \text{DCC}) - 0.09$. Storage time of the cytosol did not significantly affect the LH-20 estimate (partial correlation coefficient, $r = -0.04$).

All tumour cytosols found to be E_2R negative when assayed by one method were also found to be E_2R negative by the other method and in no case to date have the methods failed to agree as to whether a cytosol was E_2R positive or negative, although in one instance where the DCC method gave an E_2R value of 10 fmole/mg, the gel

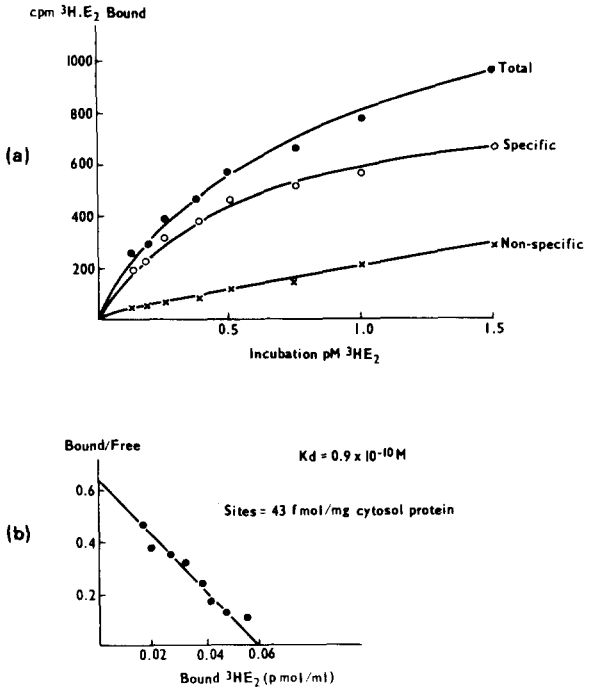

Fig. 1. (*a*) *Binding of* $^3H.E_2$ *by receptor + ve breast tumour cytosol, determined by DCC method.*
(*b*) *Scatchard analysis of data*

filtration method estimate was inconclusive. The mean dissociation constant (K_d) of the complex for 65 tumour cytosols determined by the DCC method was 2.2 ± 0.2 (S.E.M.) $\times 10^{-10}$ M. All except 2 values (8.3 and 8.7×10^{-10} M respectively) fell in the range $0.5–5.0 \times 10^{-10}$ M. The mean K_d for 24 E_2R+ tumour cytosols determined by the LH-20 method was 2.6 ± 0.3 (S.E.M.) $\times 10^{-10}$ M and all values lay in the range $0.5–5.0 \times 10^{-10}$ M. Provided that the experimental data yield a satisfactory Scatchard plot, it appears that either method has a sensitivity level of 5 fmole/mg cytosol protein.

Preliminary tests with rat uterus cytosol (subsequently confirmed with human breast tumour cytosol) indicated that the cytosol could be stored in liquid nitrogen for 2–3 weeks without appreciable loss of receptor activity provided the protein concentration was not less than 1 mg/ml protein and that a sulphydryl group preservative reagent was included in the buffer. At first, cytosols for E_2R estimation were prepared in buffer A (containing dithiothreitol). When both E_2R and PgR were measured in the same cytosol, the presence of glycerol was found to be necessary to preserve PgR stability and buffer B (containing thioglycerol) was used in preparation of the tumour cytosol. Either buffer may be used for preparation of cytosol for E_2R measurement with reproducible results.

When a cytosol which had been stored in liquid nitrogen was allowed to thaw and subsequently re-frozen in liquid nitrogen, it was found that on thawing for a second time, receptor activity was completely lost. On the other hand, a freshly prepared cytosol stored for 24 hr at 0–4°C suffered no loss of oestrogen receptor activity.

Table 1 illustrates the reproducibility of E_2R data obtained from four primary tumours by either method. The data indicate that the tumour tissue appeared to be homogeneous with regard to receptor sites and that the receptor sites were not appreciably affected by storage of the tissue in liquid nitrogen for several months or by storage of the cytosol for up to 2 weeks.

(b) *Progesterone receptors* (*PgR*). Difficulty was experienced in our early trials with ^3H-progesterone in demonstrating the presence of specific high affinity progesterone receptor in tumour cytosols since, in addition to low-affinity, high capacity non-specific binding such as is observed with oestradiol-17β, progesterone also binds to corticosteroid binding globulin (CBG) and cytoplasmic glucocorticoid receptors. The synthetic progestin R5020, used by Philibert and Raynaud [8] to identify progesterone receptor in immature rat and mouse uteri was employed by Horwitz and McGuire [12] and by Raynaud *et al.* [13] to assay progesterone binding sites in breast tumour tissue. We have used ^3H-R5020 successfully in both our assay procedures for estimation of PgR in tumour cytosols with excess non-radioactive R5020 as competitor to discriminate specific binding.

Figure 2(a) shows a representative ^3H-R5020 binding curve for a typical PgR+ cytosol. Scatchard analysis, Fig. 2(b), indicated the presence of a single class of high affinity receptor. A comparison was made between estimates of PgR activity obtained by the DCC and LH-20 methods for 15 tumour cytosols with PgR+ values ranging from 25 to 1200 fmole/mg cytosol protein. In each case, estimation by the DCC method was made on freshly prepared cytosol, that by the LH-20 method on two-thirds of the samples was made after periods of from 0.5 to 4 weeks' storage of the cytosol in liquid nitrogen. Agreement was very good. The correlation coefficient was statistically highly significant, $r = 0.93$, $P \ll 0.001$. However, it was found that the LH-20 estimate decreased with increasing storage time (partial correlation coefficient, $r = -0.65$) and taking this into consideration the coefficient of multiple correlation is $R = 0.96$, $P \ll 0.001$. The regression equation, log(PgR −LH-20) = 0.89 log(PgR − DCC) − 0.09 in-

Table 1. Comparison of E_2R capacity of primary tumours determined under different conditions of storage and by different assay methods

Tumour		Age of cytosol (days) at assay*	mg Protein/ml cytosol	E_2R (fmole E_2 bound/mg cytosol protein) DCC method	LH-20 method
LT1	On receipt	(1) Fresh	2.8	67	34
		5		43	
		12		35	
	After 9 months in liq. N_2	(2) Fresh	1.5	28	
		(3) Fresh	1.5	44	
LT2	On receipt	(1) Fresh	2.4	22	
		3		19	
		4			16
	After 2 months in liq. N_2	(2) Fresh	2.3	20	
LT3	On receipt	(1) Fresh	2.0	−ve	−ve
		(2) Fresh	3.3	−ve	
LT4	On receipt	(1) Fresh	2.9	168	129
		5		138	
		7		172	
	After 1 week in liq. N_2	(2) Fresh	3.3	124	
		1		136	
		3			104
		8			98

*Stored in liquid N_2.

terval (weeks) + 0.28, represents this relationship.

All tumour cytosols found to be lacking in PgR when assayed by the DCC method were also found to be PgR − when the LH-20 technique was employed and in only one instance was an inconclusive result obtained by the LH-20 method where the DCC procedure estimate was positive, a result possibly accounted for by the very high ambient temperature on the day of the LH-20 assay.

The mean dissociation constant (K^d) of the PgR complex for 27 tumour cytosols determined by the DCC method was 7.7 ± 0.7 (S.E.M.) $\times 10^{-10}$ M. Only one value, 16.6×10^{-10} M, fell outside the range $2.0–14.0 \times 10^{-10}$ M. The mean K_d for 18 cytosols determined by the LH-20 method was $7.5 \pm 0.7 \times 10^{-10}$ M and again only one value, 15.3×10^{-10} M, lay outside the range $2.0–14.0 \times 10^{-10}$ M. Given a satisfactory Scatchard analysis, either method could detect progesterone receptor down to a level of 15 fmole/mg cytosol protein.

Available data on the stability of progesterone receptor in cytosol stored in liquid nitrogen is at present insufficient and inconclusive. On the other hand, we have evidence which suggests there is no appreciable loss of progesterone receptor activity in tumour tissue stored in liquid nitrogen for up to 4 months.

Cytosols stored in liquid nitrogen, allowed to thaw and re-frozen in liquid nitrogen were found to have lost all progesterone receptor activity on subsequent thawing.

Incidence of E_2R+ and PgR+ in primary and metastatic tumours

Of 71 primary mammary tumours examined for oestrogen receptor activity, 44 were found to be E_2R+ (62.0%), amounts of E_2 bound ranging

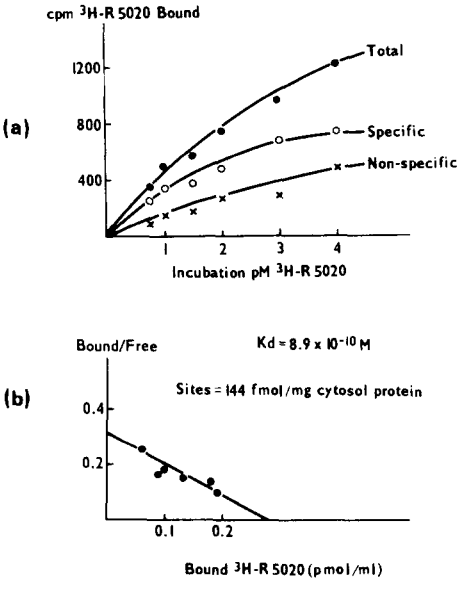

Fig. 2. (a) Binding of progestin ^3H.R5020 by receptor + ve breast tumour cytosol, determined by DCC method.

(b) Scatchard analysis of data.

from 8 to 650 fmole/mg cytosol protein. Progesterone receptor assay was also performed on 54 of these samples, 22 of which were found to be PgR + (40.7%) with amounts of R5020 bound varying from 17 to 1200 fmole/mg cytosol protein. Twenty-two out of thirty-five (62.8%) of tumours which were E_2R + were also PgR +. In no instance was PgR + activity found in a tumour which was E_2R −.

Of 43 secondary tumours, 22 (51.2%) were found to have E_2R + activity with a range of bound oestradiol from 5 to 372 fmole/mg cytosol protein. Progesterone receptor estimation was carried out on 12 of these samples, 5 of which (41.7%) were found to have PgR + activity within a steroid-binding range of 17–145 fmole/mg protein. Seven of these twelve tumours were E_2R + so that 71.4% (5/7) were positive for both activities. In no instance was PgR + activity found in a tumour which was E_2R −.

The results obtained for these tumours which were both E_2R + and PgR + are shown in Fig. 3. It is apparent that the two receptors are not found in constant proportions. In five of the primary tumours, a higher concentration of oestradiol receptor was present, in the remaining 17 a higher proportion of progesterone receptor was found. Of the 5 metastases which contained both receptors, the oestradiol receptor content was the higher in two cases, with the progesterone receptor content prevailing in the other three samples.

In Fig. 4, E_2R binding capacity determined in tumours is related to patients' ages. The mean value in premenopausal women was found to be considerably less than in postmenopausal subjects, and the proportion of E_2R + to E_2R − in the tumours of the former (40%) was less than in those of the latter group (67.1%). The scatter diagram indicates a trend towards higher E_2R binding capacity with increasing age. The correlation coefficient is statistically significant: $r = 0.37$, $P < 0.01$. PgR binding capacity in tumours does not appear to correlate with the patient's age (Fig. 5. $r = -0.08$).

Correlation of clinical response with oestrogen receptors

Thirty patients with metastatic breast cancer have been treated with various forms of hormone therapy. The minimum follow-up has been 7 months and the longest 1 yr.

Before starting therapy, accessible metastatic lesions were biopsied. Half of the material was sent for histological examination and the re-

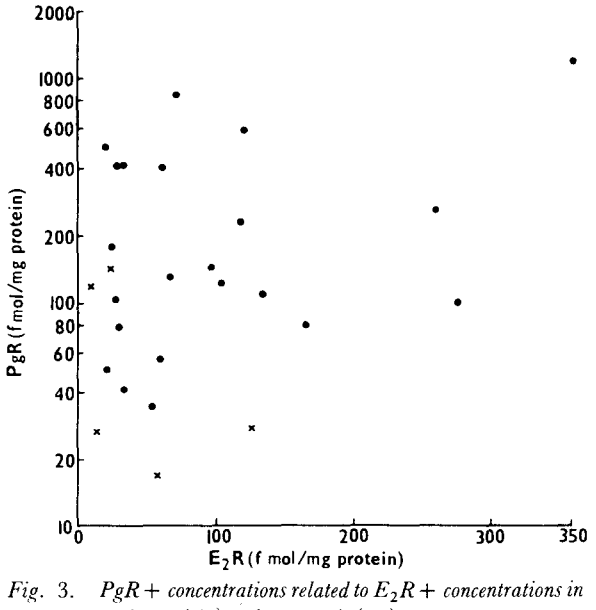

Fig. 3. PgR + concentrations related to E_2R + concentrations in primary (●) and metastatic (×) tumours.

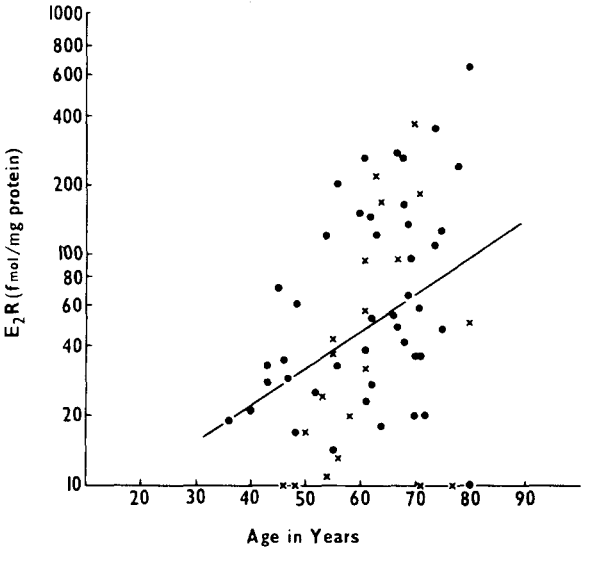

Fig. 4. E_2R + concentrations in primary (●) and metastatic (×) tumours related to patients' ages.

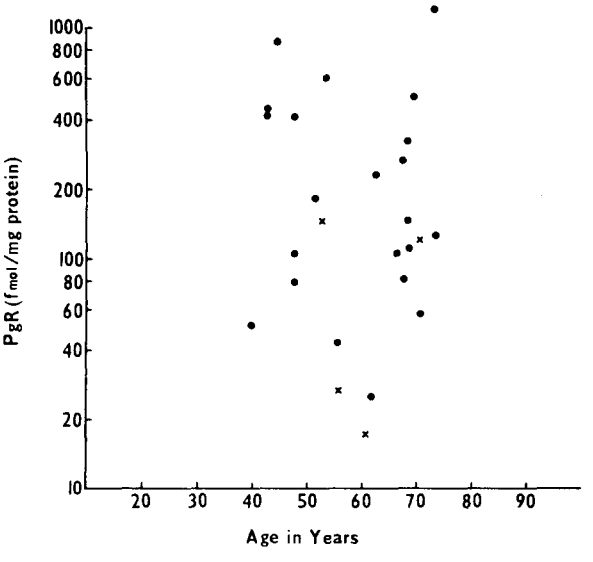

Fig. 5. PgR + concentrations in primary (●) and metastatic (×) tumours related to patients' ages.

mainder used for receptor assay. Careful clinical examinations were made and each patient had accurate measurements of visible and palpable lesions. Clinical photographs were taken. A full blood count and biochemical profile was done and plasma 17β oestradiol assay was also carried out on patients before additive hormone therapy was given. A chest X-ray and X-rays of the major portions of the skeleton were carried out and whenever possible a whole body TC99 scan.

Assessment of response. The criteria laid down by the British Breast Group [14] have been followed. Assessment was made at 6 months from the start of therapy.

Table 2 shows the correlation of response to hormone therapy for 17 patients with metastatic breast tumour classed as being E_2R+.

Table 2. E_2R+ metastatic tumours

Type of therapy	Objective	Response Partial	Failure
Tamoxifen	5/11	4/11	2/11
Diethyl stilboestrol	1/5	1/5	3/5
Prednisolone	0/1	0/1	1/1
Total	6/17	5/17	6/17

Using the criteria above, 6 patients had an objective response and 5 a partial response. There were 6 failures. The total response was approximately 62%. Of the 6 failures, 3 patients had more than one form of therapy followed by cytotoxic drugs but still no response was obtained. These 3 patients are listed in the Table under the first hormone used. The patients were all postmenopausal, ranging from 6 months to more than 10 yr postmenopausal. Where the disease responded objectively, in all cases it was soft tissue disease that regressed, i.e. disease in the operative flaps and regional nodes. The plasma 17β oestradiol in all the patients was so low as to be virtually undetectable.

In Table 3 is shown the response to therapy in 13 patients with E_2R- metastatic tumours. Only 1 patient has had the disease on the chest wall and lung metastases static for 6 months. This is classed as a partial response, since the disease was progressing prior to hormone therapy. Again the patients were all postmenopausal. Two patients did not respond to other hormones or to cytotoxic drugs.

Table 3. E_2R- metastatic tumours

Type of therapy	Objective	Response Partial	Failure
Tamoxifen	0/6	1/6	5/6
Androgens	0/2	0/2	2/2
Prednisolone	0/2	0/2	2/2
Progestogen	0/1	0/1	1/1
Bilat. oophorectomy	0/2	0/2	2/2
Total	0/13	1/13	12/13

DISCUSSION

Two simple quantitative methods, one based on the dextran–charcoal procedure of Korenman and Dukes [6] and the other using gel filtration on Sephadex KH-20 as originally described by Ginsburg *et al.* [7] for measurement of oestradiol receptors in brain and pituitary, have been used to estimate both E_2R and PgR activity in the same tumour cytosol. Where adequate control over experimental parameters (especially temperature) and storage conditions was exercised, agreement between the assay estimations of either receptor activity was remarkably good and in no instance did the methods fail to agree on whether a tumour cytosol was positive or negative in respect of either receptor. The K_d values observed provide good evidence for the specificity and identity of the individual receptors measured by either method. Either procedure has a sensitivity level of 5 fmole/mg cytosol protein for E_2R and 15 fmole/mg cytosol protein for PgR, and these levels have therefore been regarded as the cut-off points for determining whether a tumour was receptor positive, subject to a satisfactory Scatchard analysis being obtained. Use of either method would permit 15–20 assays of both receptors to be carried out in a normal working week by a single operator.

Addition of glycerol and thioglycerol to the homogenisation buffer (Jänne *et al.* [15]; Horwitz and McGuire [12]) greatly increased the stability of the progesterone receptor and did not affect subsequent estimation of E_2R so that the same tumour cytosol prepared in 30% glycerol may be used for assay of both activities.

Under the assay conditions described, the E_2R assay measures predominantly free, unoccupied receptors [16]. As shown by Sakai and Saez [17], however, binding sites in all tumours are undersaturated by oestradiol *in vivo* and the absolute number of receptors occupied by endogenous oestradiol (which rarely exceeds 15% in postmenopausal or 20% in premenopausal women) is partly dependent on the total numbers of receptors available. Because of its greater affinity for the progesterone receptor, ^{3}H-5020 as used in the PgR assay measures not

only unoccupied sites but also occupied sites from which it displaces progesterone [13].

Various authors have reported positive E_2R values in proportions ranging from 50 to 80% of primary tumours assayed and there is general agreement that there is a lower incidence of E_2R+ in metastatic specimens [2]. In our series to date, we have found an incidence of 62% positive results in primary, 51% in metastatic tumours. The proportion of E_2R+ in the tumours of premenopausal women (40%) was markedly less than in those of postmenopausal subjects (67%), and we also found the mean number of sites in the younger women with E_2R+ to be lower than in the older age groups, in keeping with the data reported by other groups on the relationship between E_2R+ tumours and patient's age and menopausal status (see McGuire *et al.* [2]).

In the group of 30 postmenopausal patients with metastatic breast cancer studied we found 62% of 17 E_2R+ cases responded to hormone manipulation, whereas 12 out of the 13 E_2R- cases failed to respond and in the remaining patient the disease remained static. Our data therefore agree with the collective experience of several centres reported by McGuire *et al.* [2]: patients with breast cancer who lack oestrogen receptors in their tumour tissue have a minimal prospect of responding to endocrine therapy, but some 40% of these with E_2R+ tumours fail to respond to hormone manipulation. This would suggest that although the cells in this latter group of tumours are capable of the initial step of binding oestrogens, there is a lesion at some later stage in the chain of hormone action which prevents them from responding to changes in the hormonal environment. To identify the hormone responsive tumours more precisely therefore requires measurement of some later product of oestrogen action in the cell.

Freifeld *et al.* [18] and Reel and Shih [5] demonstrated the induction of progesterone receptor activity in uterine tissue following oestradiol administration. Horwitz *et al.* [4] have postulated that the presence of PgR in a tumour would therefore indicate that the tumour was capable of synthesizing at least one end product under oestrogen regulation and hence might be expected to be responsive to hormone therapy.

Only a few reports have so far appeared in the literature of the incidence of PgR in human breast cancer (Terenius [3]; Horwitz *et al.* [12], Raynaud *et al.* [13], Liskowski *et al.* [19]). This probably stems from the difficulty of identification of specific PgR in the presence of corticosteroid-binding globulin and cytoplasmic glucocorticoid receptors for which progesterone has marked affinity and from the unstable nature of the PgR complex. This difficulty has been overcome by the use of the radioactive synthetic progestin R5020 which was used by Philibert and Raynaud [8] with immature rat and mouse uteri and by Horwitz and McGuire [12] with human mammary carcinoma to demonstrate the presence of specific PgR.

McGuire's hypothesis [4] requires that PgR should be rare in tumours where E_2R is absent. All E_2R- tumours which we have examined to date (19 primary, 5 metastatic) have been found to have no PgR activity. Forty-one to forty-two percent of all tumours (primary and metastatic) on which PgR estimation was carried out were found to have PgR+ activity. These results are in marked contrast to the data published by Liskowski *et al.* [19] who report PgR+ in 85% of all tumours assayed and 40% of these classified as E_2R- (i.e. <10 fmole E_2 bound/mg cytosol protein). This would suggest that the type of assay used by these workers which employs ^3H progesterone at a single concentration is failing to discriminate specific PgR activity effectively. Sixty-three to sixty-four percent of all tumours (primary and secondary) on which we have carried out both receptor measurements and which have been found to be E_2R+ have also PgR+ activity, which accords well with McGuire's hypothesis that possession of PgR+ activity should indicate which of approximately 60% of tumours with E_2R+ have retained hormone dependence and might therefore be expected to respond to hormone therapy. Although our data on the clinical response of patients with metastatic breast cancer is at present limited to 30 patients whose tumour E_2R activity was estimated prior to treatment, the overall response rate (62%) is consistent with the percentage of all E_2R+ tumours in which we have also found PgR+ activity. Of seven patients whose tumour PgR was also estimated, three with PgR+ had a remission, four with PgR− failed to respond.

In agreement with Liskowski *et al.* [19] we found no linear correlation between estimated values for E_2R and PgR in primary and metastatic breast tumours. Unlike oestradiol binding capacity, which shows a trend towards higher levels with increasing age and change in menopausal status, progesterone binding capacity does not appear to bear any relationship to the patient's age.

In our experience, limited to response in menopausal patients with metastatic breast cancer, there does not appear to be any direct relationship between the absolute value for tumour E_2R and the percentage response. In

selecting suitable standardized assay systems for estimating both E_2R and PgR in tumours, it seemed to us more important that each assay should have a clearly defined threshold value which distinguished between positive and negative binding and that from the data in each individual assay it should be possible to demonstrate that the cytosol contained only one class of binding sites whose dissociation constant conformed to that expected for the specific tissue receptor assayed. When these criteria are observed, we conclude that an accurate picture of the tumour's responsiveness to hormones should be obtained. We await data which it is hoped will provide direct correlation between clinical response and presence or absence of PgR, but our present evidence would appear to support the hypothesis of Horwitz *et al.* [4] that the presence of both PgR and E_2R would indicate a tumour whose endocrine regulatory mechanism was largely intact and which might therefore be more confidently predicted to respond to endocrine therapy. If this were indeed the case, estimation of E_2R and PgR in primary tumours at time of mastectomy might serve as a useful guide to predict response to hormone therapy when metastases appear at a later time, (often in inaccessible sites), as suggested by Jensen *et al.* [20]. This is at present under investigation in our laboratory.

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Cell Surface Charge, Sialic Acid Content and Metabolic Behaviour of Two Tumour Sublines. A Comparative Study*

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Abstract—*Two sublines of the Ehrlich-Lettré mouse ascites tumour, the glycogen-storing and the glycogen-free strains, were compared with respect to their respiratory and glycolytic activities, their electrophoretic mobility and the cell surface sialic acid content. An attempt to correlate differences in these parameters with the growth behaviour of the two cell types showed some deviations from "classical" malignancy criteria. Although the two cell lines exhibited differences in their surface sialic acid content we did not find similar differences in reduction of electrophoretic mobility after neuraminidase treatment.*

INTRODUCTION

THERE has been considerable interest in the electrical charge of the cell surface, especially with respect to malignancy, ever since Ambrose *et al.* [1] advanced the hypothesis that malignant transformation is associated with elevated net negative cell surface charge. It has been proposed that neuraminic acids may mask cell surface antigens [2–5]. Much significance is attributed to the surface charge for phenomena like invasiveness, metastasis and contact inhibition of growth [6]. On the other hand, attempts to correlate membrane sialic acid content directly with anodic electrophoretic mobility have met with little success [7–9]. In addition to sialic acids, membrane-associated ribonucleic acids [10, 11], sulphate and thiol groups [12] are believed by some authors to contribute to the net negative surface charge. In the present study we compared closely related tumour sublines with different biochemical and growth characteristics with special regard to surface charge and sialic acid content. We chose two strains of the hyperdiploid Ehrlich–Lettré ascites tumour (EAT) which have developed spontaneously from the same original tumour: one subline (G+) stores glycogen, especially in the nucleus, whereas the other one (G∅) is essentially free of glycogen deposits [13]. The two variants differ in several biological, biochemical and pharmacological properties. For example, the G+ strain exhibits a faster growth rate; glucose is primarily utilized for glycogen synthesis (with the G∅ strain for lipid biosynthesis); the G+ cells are partially resistant against antitubulins such as colchicine and vinca alkaloids [14, 15]. Since such dissimilarities point to a possible involvement of the cell surface it seemed promising to compare the two sublines in terms of their surface charge and sialic acid content, as well as their metabolic characteristics, and to try to correlate these parameters with the growth behaviour. Preliminary results have already been reported [16].

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MATERIAL AND METHODS

(*a*) *Cells*

The glycogen-free (G∅) and glycogen-storing (G+) sublines [14] of the hyperdiploid Ehrlich-Lettré mouse ascites tumour (EAT) were grown in male NMRI mice weighing about 30 g. The cells were harvested 7 days after inoculation, suspended in cold Hank's solution, centrifuged 10 min at 120 × ***g***, and, depending on the nature of the subsequent experiment, washed again in the same way up to 3 times, or resuspended immediately in an appropriate buffer system.

(*b*) *Metabolic assays*

The determination of global metabolic parameters, i.e. respiratory and glycolytic rates, with and without exogenous substrates (10 mM glucose) was carried out with four independent

methods as described earlier [17, 18]: 1. classical Warburg manometry; 2. enzymatic determination of lactate production; 3. autotitration in pH-stat systems under anaerobic conditions (N_2); 4. continuous automatic measurement of oxygen consumption under constant oxygen pressure using a combined polarographic–titrimetric method. As we did in previous studies [17, 18], we compared these methods for each experimental condition. Again we found a high degree of congruence between the different methods.

(*c*) *Determination of electrophoretic mobility of cells*

We used the cytopherometer from Zeiss, Oberkochen (Germany), developed by Ruhenstroth-Bauer and Fuhrmann [19]. The measuring chamber is connected to a Zeiss phase contrast microscope, and the electrophoretic mobility of cells is expressed in (μ/s)/(V/cm). All mobility measurements were carried out at 25 $\pm$0.5°C and repeated with reversed polarity. In each experiment, the mobility of at least 40 cells was determined. We used the following composition for the measuring fluid: 7.5 ml isotonic sucrose solution, 2.0 ml phosphate buffer, pH 7.18, 300 mOsM made up to 10.0 ml with 0.5 ml cell suspension, the latter being diluted 1:4 with physiological saline. We chose this composition as a "compromise", following an advice given by Fuhrmann (Fuhrmann, personal communication): on the one hand the medium should possess a relatively high buoyant density in order to facilitate mobility measurements, on the other hand the percentage of ion-free liquid should not exceed 80% since the properties of the cell surface may be altered (cf. [20]). The final cell concentration was about 3×10^4/ml.

(*d*) *Neuraminidase treatment of cells and sialic acid determination*

Washed cells were subjected to treatment with *Vibrio cholerae* neuraminidase (glycoprotein N-acylneuraminyl hydrolase, E.C. 3.2.1.18.) from Behringwerke, Marburg. The optimum enzyme concentration and incubation time had to be found for our purposes in order to release sufficiently high amounts of sialic acid with incubation times during which the cells do not lose their functional integrity. We compared incubation times from 15 min to 6 hr, with enzyme concentrations of 25, 50 and 100 U*/ml cell suspension and 6×10^6 cells/ml. We found that 50 U/ml for 60 min yielded the best results. Using this procedure, more than 90% of sialic acids liberated with 100 U/ml within 6 hr are released. Similar results have been obtained by Kojima and Maekawa [9]. Unless stated otherwise, this protocol was applied for electrophoretic studies as well as for the determination of cell surface sialic acid. The following buffer system was used throughout: 25 ml maleic acid in Tris (24.23 g Tris hydroxyaminomethane plus 23.21 g sodium maleate per liter of twice distilled water); 17.9 ml 0.2 N NaOH; 100 mg $CaCl_2$, made up to 100 ml with twice distilled water. The osmolarity was adjusted to 290 mOsM; the pH was 6.5. Nine-tenths of a millilitre of this maleate buffer was mixed with 1.0 ml of washed cells and 0.1 ml of neuraminidase, containing 100 U. The incubation was carried out at 37°C while the cells were gently shaking in Warburg flasks. After incubation, the cells were centrifuged at $120 \times g$ for 15 min and resuspended to the original volume in physiologic saline containing 10^{-2} M EDTA. Omission of the latter sometimes resulted in aggregation of cells which made cytophoretic studies impossible. The supernatants were used for the determination of sialic acid according to the method of Warren [21] (acidic oxidation of free sialic acids and colorimetric determination of the coloured complex produced with 2-thiobarbituric acid).

(*e*) *Assessment of membrane integrity of treated cells*

Prior to cytophoretic examination, neuraminidase-treated cells were routinely examined in the phase-contrast microscope. In some cases, electron micrographs were kindly made by Prof. Forssmann (Dept. of Anatomy). Furthermore, leakage of cytoplasmic lactate dehydrogenase (E.C. 1.1.1.27.), as determined photometrically, and of potassium ions, measured with an atomic absorption spectrophotometer (Pye-Unicam SP 90), as well as the trypan-blue exclusion test were used for the assessment of membrane integrity. Cell "viability" was determined with a laser flow cytofluorometer, with fluorescein diacetate as "fluorogenic substrate", cf. [22], for intact cells and erythrosine B as fluorescent counterstain for damaged cells. With 50 U neuraminidase/ml for 60 min at 37°C, about 90% of the cells were functionally "intact" as judged by these criteria.

RESULTS

(*a*) *Metabolism*

As can be seen from Fig. 1, left side, the

*1U is defined as the amount of neuraminidase which releases 1 μg of N-acetylneuraminic acid from human α-acid glycoprotein in 15 min at 37°C, in a sodium acetate buffer solution 0.05 M, pH 5.5, containing 9 mg NaCl/ml and 1 mg $CaCl_2$/ml (producer's specification).

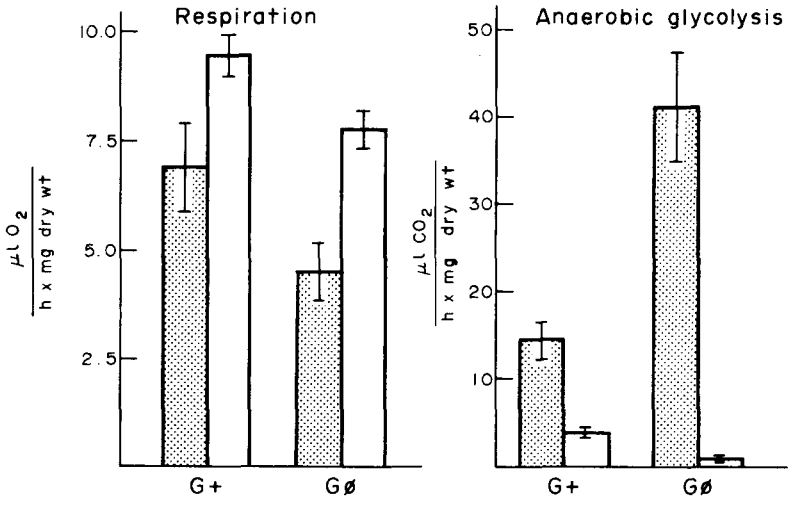

Fig. 1. Metabolic rates of glycogen-storing (G+) and glycogen-free (GØ) Ehrlich–Lettré mouse ascites tumour cells. Left side: respiratory activity after addition of 10 mM *glucose (dark columns). Right side: anaerobic glycolysis with* 10 mM *glucose (dark columns) and without exogeneous substrate (light columns). Respiratory activity is expressed in* µl O_2 *consumption per* hr *and* mg *cell dry mass, anaerobic lactate production in* µl CO_2 *liberated from the bicarbonate buffer system per* hr *and* mg *cell dry mass.* $n = 14$.

glycogen-storing cells (G+) show a considerably higher O_2-consumption than the GØ cells when exogeneous substrate (10 mM glucose) is supplied. Without the addition of glucose, both cell types exhibit increased respiratory activity when the determination is carried out within 90 min after aspiration of the cells from the peritoneal cavity. It also can be seen that the impairment of respiration in the presence of glucose, a phenomenon known as "Crabtree effect" [23], is more pronounced with the GØ strain. In contrast, the glycolytic activity under anaerobic conditions is considerably higher with the GØ cells (Fig. 1, right side). Interestingly, the glycogen-storing cells show the ability to perform anaerobic glycolysis to some extent even in the absence of exogenous glucose for about one hour.

(*b*) *Electrophoretic mobility*

Figure 2 (left side) shows that the electrophoretic mobility of the G+ cells is higher. This finding applies, however, only to cells which have been washed once immediately after aspiration. When the cells were subjected to shaking in Warburg flasks for 60 min, this difference in electrophoretic mobility disappeared.

(*c*) *Cell surface sialic acid content*

Figure 2 (right panel) depicts the amount of

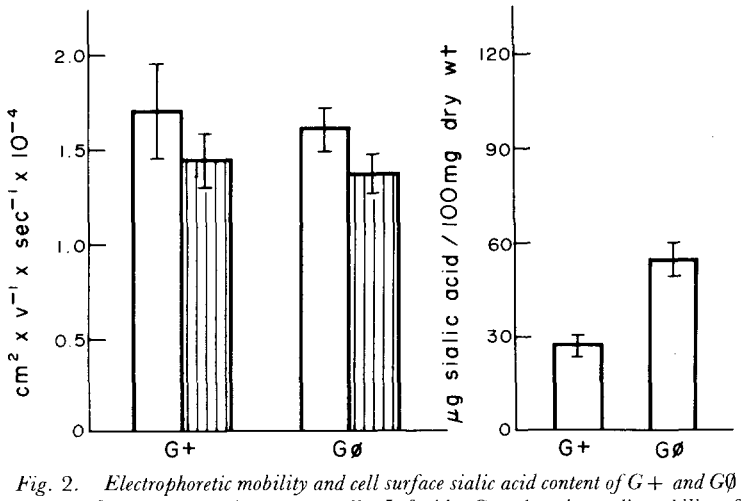

Fig. 2. Electrophoretic mobility and cell surface sialic acid content of G+ and GØ Ehrlich–Lettré mouse ascites tumour cells. Left side: Cytophoretic anodic mobility of native cells (light columns) and of cells treated with 50 U Vibrio cholerae *neuraminidase/*ml *for* 60 min *at* 37 °C *(dark columns). The mobility is expressed in* (µ/s)/(V/cm). *Right side: Sialic acid content, as determined by the thiobarbituric acid assay.*

sialic acids removable by neuraminidase from the two cell types. It seems of particular interest that the GØ cells contain much more, i.e. almost twice as much, sialic acid susceptible to ketosidic cleavage by neuraminidase than the glycogen-storing ones, although the cytophoretic behaviour (left panel) might rather suggest the contrary.

(d) Electrophoretic mobility after neuraminidase treatment

After neuraminidase treatment (50 U/ml, 60 min, 37°C), the electrophoretic mobility for GØ cells was found to be:

$1.37 \pm 0.09 \frac{\mu/s}{V/cm}$, as compared to

$1.61 \pm 0.12 \frac{\mu/s}{V/cm}$ for untreated cells.

For G+ cells the corresponding values were:

$1.45 \pm 0.14 \frac{\mu/s}{V/cm}$ (treated) and

$1.80 \pm 0.26 \frac{\mu/s}{V/cm}$ (untreated) (Fig. 2, left panel). This means a decrease in electrophoretic mobility by 15% for GØ, and 19% for G+ cells, respectively. These values are considerably lower than those reported in the literature for Ehrlich and other ascites cells [7–9, 24]. The puzzling inconsistency with the values for neuraminidase-liberated sialic acids (right panel in Fig. 2) remains to be discussed.

DISCUSSION

It always has been a problem to establish "malignancy criteria" other than growth rate, invasiveness or degree of dedifferentiation of specialized cells. With the data of this comparative study on two tumour sublines no definite correlation can be obtained between known differences in growth rate [14], "classical" matabolic malignancy criteria [23, 25], "malignant" electrokinetic behaviour [1] and sialic acid content of the cell surface.

The faster-growing glycogen-storing cells exhibit a higher respiratory activity, with and without addition of exogenous glucose, and a considerably lower glycolytic rate under anaerobic conditions (Fig. 2). In addition, reduction of respiratory activity in the presence of glucose, the so-called "Crabtree effect" [23], is more pronounced with the glycogen-free cells (dark columns in Fig. 2, left panel). The decrease in O_2-consumption is parallelled by a rise in aerobic lactate production (not shown on the graph). All these findings would suggest that the GØ strain is "more malignant", according to "classical" metabolic criteria. On the other hand, we were able to demonstrate that the glycogen-storing cells are capable to maintain about 30% of their anaerobic glycolytic rate for about 1 hr in the absence of glucose, an ability lacking with GØ cells. Apparently the stored glycogen can be mobilized at times of nutritional deficiency, and in fact, microscopical examination of such cells revealed the disappearance of most of the cytoplasmic and many of the intranuclear glycogen grana as compared with control cells maintained under the same conditions with exogeneous glucose. One might speculate that this possibility offers an advantage for the *in vivo* growth of G+ cells which makes these cells, at least temporarily, independent of exogenous substrates, especially when they are remote from adequate oxygen and substrate supply.

The interpretation of cytophoretic mobility is quite intricate, some of the difficulties have been reviewed recently [26]. Yet we think that cytophoresis is a useful means to estimate cell surfaces-charge properties for comparative purposes. Although other charge carriers are being discussed, we believe that for cells growing in suspension the bulk of ionogenic groups that generate the net negative cell surface charge is contributed by sialic acids, in spite of the controversial findings discussed below. It has been demonstrated by others that sialic acids are responsible for electrostatic repulsion between cells, cf. [27]. Moreover, there is evidence for the existence of membrane-associated "ecto-sialidases" [28, 29], a finding that might be of great importance for cell–cell interactions and tumour biology. If Ambrose's [1] hypothesis is applied to our cells, the G+ strain with its greater anodic electrophoretic mobility (Fig. 2) ought to be the "more malignant" one. On the other hand, it has been found with normal and proliferating liver cells that the latter exhibit an increase in electrophoretic mobility to about the same extent as malignant liver cells [19]. Thus it has been proposed that higher negative surface charge relates to faster growth. This is consistent with the higher growth rate of G+ cells, as compared with the GØ strain [14], whether this means "greater malignancy" or not. It should be pointed out that we found electrophoretic mobilities for both cell types that are higher than those reported earlier for Ehrlich cells by Hoelzl Wallach *et al.* [7, 8] and Mayhew [24]. On the other hand, the reduction in electrophoretic mobility after neuraminidase treatment was markedly smaller (19% for G+ cells) than

reported by Hoelzl Wallach (72%). The inverse relationship between electrokinetic behaviour and amount of enzyme-removable Warren-positive material is not unique. Similar discrepancies have been encountered by other investigators, cf. [7, 8]. Possible explanations may relate to different spatial arrangements of sialic acid molecules within the membrane. Since the zeta-potential is supposed to comprise only those ionogenic groups that do not extend more than 10 Å from the hydrodynamic plane of shear, sialic acid residues located farther away from this plane would not be detected; yet they may be accessible to neuraminidase. We are currently investigating this possibility with the aid of a new preparative isoelectric focussing technique. With such a technique it should be possible to detect charge changes which occur farther away than 10 Å from the hydrodynamic plane of shear. Indeed, preliminary findings indicate that comparative studies with the two techniques allow for this conclusion [30]. On the other hand, removal of sialic acids may lead to a conformational change within the membrane, giving rise to the appearance or disappearance of positive or negative ionogenic groups, with different results for different cell types. It has been pointed out in "results" that the differences in electrokinetic behaviour between the two cell types disappear after shaking for 60 min. This raises the possibility that even mechanical events may lead to membrane conformational changes with subsequent rearrangement of ionogenic groups. Alternatively, the possibility must be considered that substituted sialic acids may be present other than N-acetyl-D-neuraminic acid which may not be substrates for *Vibrio cholerae* neuraminidase. Any one or all of these possibilities may reconcile the apparent contradiction between the amount of sialic acids liberated and the reduction in electrophoretic mobility, especially with the G+ cells.

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Modification of Tumour Cells by Covalent Attachment of *N*-Acetyl-D-Neuraminic Acid to the Cell Surface*

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Abstract—*Two sublines of the Ehrlich-Lettré mouse ascites tumour were compared with respect to their ability to covalently bind the 4′-diazobenzyl-α-ketoside of N-acetyl-D-neuraminic acid* per viam *azo linkages. The increase in anodic mobility of the cells was correlated with their content in neuraminidase-removable sialic acid before and after the coupling procedure.*

INTRODUCTION

TWO SUBLINES of the Ehrlich-Lettré mouse ascites tumour, glycogen-storing (G+), and glycogen-free (GØ) were compared with respect to their "responsiveness" to accept "artificially grafted" sialyl residues, which, as diazonium salts, are covalently attached chiefly to tyrosyl and histidyl residues.

To our knowledge, chemical attachment of sialic acids to the surfaces of viable cells has not been performed before. Preliminary results have already been reported [1]. In addition, we performed similar attachment reactions under mild conditions with erythrocytes [2, 3] and with proteins, having defined biological activity [4].

Although such additional negatively charged groups are located on protein moieties rather than on the oligosaccharide chains of the cell surface, such "grafting" experiments seem quite attractive. They might tell us something about the relationships between cell-surface sialic acids and the specific requirements for their location with respect to the electrokinetic behaviour and growth characteristics of such cells.

MATERIAL AND METHODS

Details on the cell strains used, on the methodology for metabolic assays, cytophoresis, sialic acid determination and "viability" tests are given in the preceding article [5].

The 4′-aminobenzyl-α-ketoside of *N*-acetyl-D-neuraminic acid was prepared by Koenigs–Knorr glycoside synthesis. A similar approach has been used by Gielen and Uhlenbruck [6], but our method [7, 8], which yields the pure α-anomeric form, showed some major deviations from their procedure. For the preparation of the 4′-diazobenzyl derivative, the 4′-aminobenzyl compound was diazotized with sodium nitrite.

After neuralization (pH electrode) the solution was brought into Tris-HCl buffer, pH 8.2, containing about 5×10^6 cells/ml, to yield a final concentration of 3.8×10^{-3} M diazo compound. Although no unreacted nitrite could be detected nitrite under the conditions employed, we prophylactically added small amounts of urea to the neuralized solution. The reaction with the cells was carried out for 30 min at 20°C, while the cells were shaken with an amplitude of 5 cm at a frequency of 100/min. Unreacted ketoside was largely removed by washing the cells twice in PBS. Nevertheless, for the determination of neuraminidase-sensitive material, we used controls treated with the 4′-aminobenzyl-α-ketoside in order to eliminate non-covalent adsorption from the evaluation.

Treatment of the cells under the conditions described did not significantly impair their metabolic activity (respiration, glycolysis) or their "viability", as determined cytofluorometrically with fluorescein diacetate or erythrosine B, cf. [9].

RESULTS

Figure 1 summarizes the effects on glycogen-storing (G+) and glycogen-free (GØ) Ehrlich–

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Lettré mouse ascites tumour cells brought about by covalent attachment of the 4′-diazobenzyl-α-ketoside of *N*-acetyl-D-neuraminic acid, both with and without neuraminidase treatment. The left panel shows the electrophoretic mobilities, as determined by cell electrophoresis. It can be seen that the relative increase in anodic mobility is greater with the G+ subline (15%), as compared to 7% with the GØ cells. Untreated GØ cells possess a slightly lesser mobility before and after incubation with neuraminidase as compared with G+ cells. Unfortunately it could not be demonstrated whether neuraminidase treatment after azo coupling reduces the increased anodic mobility, since cells subjected to both procedures were partially damaged and not suited for cytophoretic studies.

charged sialyl residues to the cell surfaces brings about a parallel increase in both electrophoretic mobility and in neuraminidase-removable sialic acids with the two cell strains. This probably means that the "artificially" attached sialyl residues are located in membrane portions which—in contrast to their physiologically located counterparts—contribute rather uniformly to the zeta-potential, with concomitantly uniform accessibility to the enzyme.

The differences between the two cell types in the number of additionally attached sialyl residues may relate to the fact that one of the predominant factors for the "responsiveness" of the cells to our coupling reagent must be the availability of tyrosyl and histidyl residues. In other words, it seems reasonable to assume that

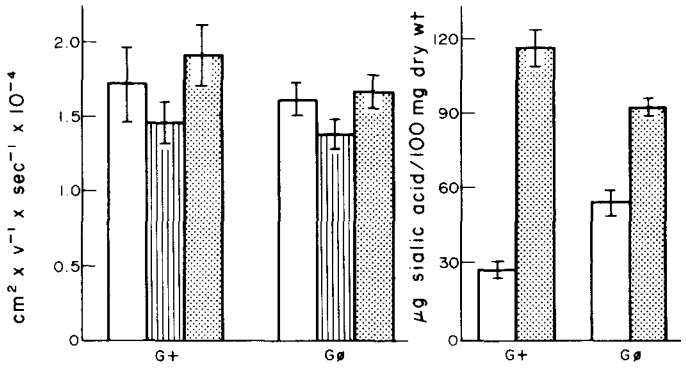

Fig. 1. Electrophoretic mobility and cell surface sialic acid content of glycogen-storing (G+) and glycogen-free (GØ) Ehrlich–Lettré mouse ascites tumour cells. Left side: Cytophoretic anodic mobility of native cells (light columns), cells treated with 50 U/ml Vibrio cholerae *neuraminidase for* 60 min *at* 37°C *(dark columns), and of cells treated with* 3.8×10^{-3} M 4′-*diazo-benzyl-α-ketoside of N-acetyl-D-neuraminic acid for* 30 min *at* 20°C, pH 8.2 *(dotted columns).* $n = 14$. *Right side: Sialic acid content, as determined by the thiobarbituric acid assay after incubation with neuraminidase* (50 U/ml, 60 min *at* 37°C). *Light columns: native cells; dotted columns: cells pretreated with* 3.8×10^{-3} M 4′-*diazobenzyl-α-ketoside as described above.* $n = 14$.

The right panel in Fig. 1 depicts the amount of neuraminidase-removable Warren-positive material: the increase after treatment with the diazobenzyl compound is considerably higher with the G+ than with the GØ subline whereas the content of neuraminidase-removable material with native cells is inversely related to the amount of additionally "grafted" sialic acid residues.

DISCUSSION

The discrepancy between the relative net negative cell surface charge and the amount of neuraminidase-sensitive material of native ascites tumour cell sublines has been one of the subjects of the preceding article [5]. On the other hand, "grafting" of additional negatively differences in covalent binding of the compound to cell surfaces may reflect different protein dispositions of the cells. Preliminary experiments with similar diazo compounds of neutral sugars and their effects on lectin binding [10] favour this view since GØ cells are, here too, less "responsive".

Since the electrokinetic behaviour of cells as revealed by cell electrophoresis is supposed to reflect ionogenic groups within only about 10 Å from the hydrodynamic plane of shear, we are currently comparing the results presented here with those obtained with a special technique for preparative isoelectric focussing of intact cells. In fact, some apparent contradictions may be resolved by using both methods [11]. Furthermore, it seems of interest to follow the electrokinetic behaviour of the cells (with either

method) when they have been subjected to neuraminidase treatment prior to coupling with the diazonium compound.

We feel that cell surface modifications with "artificially grafted", but otherwise "physiological" molecules; especially when cells of different growth behaviour are compared, may contribute to the understanding of different cell surface arrangements of whole cells. Furthermore, reimplantation experiments or similar studies with cultured cells might reveal more aspects on mutual relationships between cell surface charge and growth behaviour.

One might speculate that such reimplanted or cultured cells will repair this surface alteration brought about by the local change of negative charge. In this context it should be especially interesting to study the effect of "grafted" *N*-acetyl-L-neuraminic acid, which has not been found in nature so far. The L-isomer was synthesized in this laboratory [12–13] and found to be completely resistant to the action of neuraminidase and aldolase (*N*-acetylneuraminate pyruvate-lyase, E.C. 4.1.3.3) [13, 14]. With such L-neuraminic acid residues attached to the cell surface, a specific repair should be much more difficult. As a consequence, a modified cell would keep its higher negative charge in cell culture and after reimplantation.

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The Clinical and Histopathological Significance of Feulgen DNA-Values in Transitional Cell Carcinoma of the Human Urinary Bladder*

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Abstract—*Scanning absorption cytophotometric measurements were performed in Feulgen-stained cell nuclei in imprints from 123 biopsy specimens from human transitional cell carcinoma (TCC). The results were correlated with the histopathological grade and the clinical stage of the tumour. Nearly all histologically well differentiated bladder carcinomas and tumours of clinical stage I had diploid DNA-stemlines, while non-diploid DNA-stemlines were found in most of the dedifferentiated carcinomas and in most of the advanced cases.*

Increasing clinical stage and decreasing histological differentiation were correlated with low numbers of DNA-stemline values.

Within the group of locally advanced carcinomas, patients with non-diploid tumours had a worse prognosis than those with diploid TCC.

Distant metastases in 3 patients showed non-diploid DNA-stemlines. In some tumours the DNA-histogram showed regional differences.

INTRODUCTION

In 1948 Boivin *et al.* [1] found that the nuclear DNA-content was usually constant in cells from different tissues in the same species. A few years later it became evident that the variations in the nuclear DNA-content which can be observed in certain tissues may be explained as a result of nuclear polyploidy and by nuclear DNA-synthesis [2].

The DNA-content in carcinoma cell nuclei may differ from that of normal cells [3]. Many studies have dealt with the relationship between the nuclear DNA-content and the histopathology in human tumours. In addition, some authors have considered nuclear DNA-content in relation to clinical aspects suggesting that measurement of the nuclear DNA-content might be of prognostic value in patients with a malignant disease [4].

In this study we correlated histopathological and clinical aspects of transitional cell carcinoma (TCC) of the human urinary bladder with the results of cytophotometric DNA-measurements of individual Feulgen-stained cell nuclei in imprints from bladder tumour biopsies.

MATERIAL AND METHODS

Imprints were made from biopsies of histologically proven transitional cell carcinoma from 123 patients admitted to the Norwegian Radium Hospital during the year 1974 and from 1st October 1975 to 31st March 1976. The biopsy material ("actual biopsy") was taken from areas thought to be representative of the whole tumour, either by transurethral resection or by cystotomy. A single biopsy was taken in 118 patients, whereas both a central and a peripheral tumour area were biopsied in the remaining 5 patients (Fig. 2). In the latter the results from the

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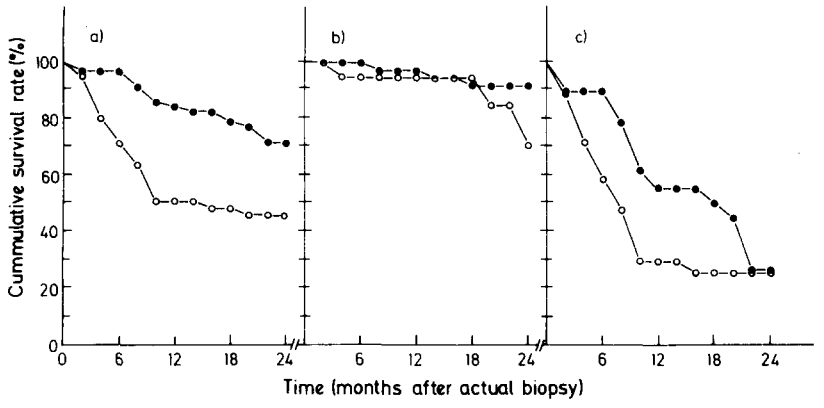

Fig. 1. Actuarial survival in patients with bladder carcinoma calculated from the actual biopsy. Tumours with diploid DNA-stemlines (● ● ●). Tumours with non-diploid DNA-stemlines (○ ○ ○). (a) All tumours (63 diploid/60 non-diploid) (b) Stage I tumours (43 diploid/20 non-diploid) (c) Stage II–III tumours (8 diploid/18 non-diploid) and Stage IV tumours (12 diploid/22 non-diploid).

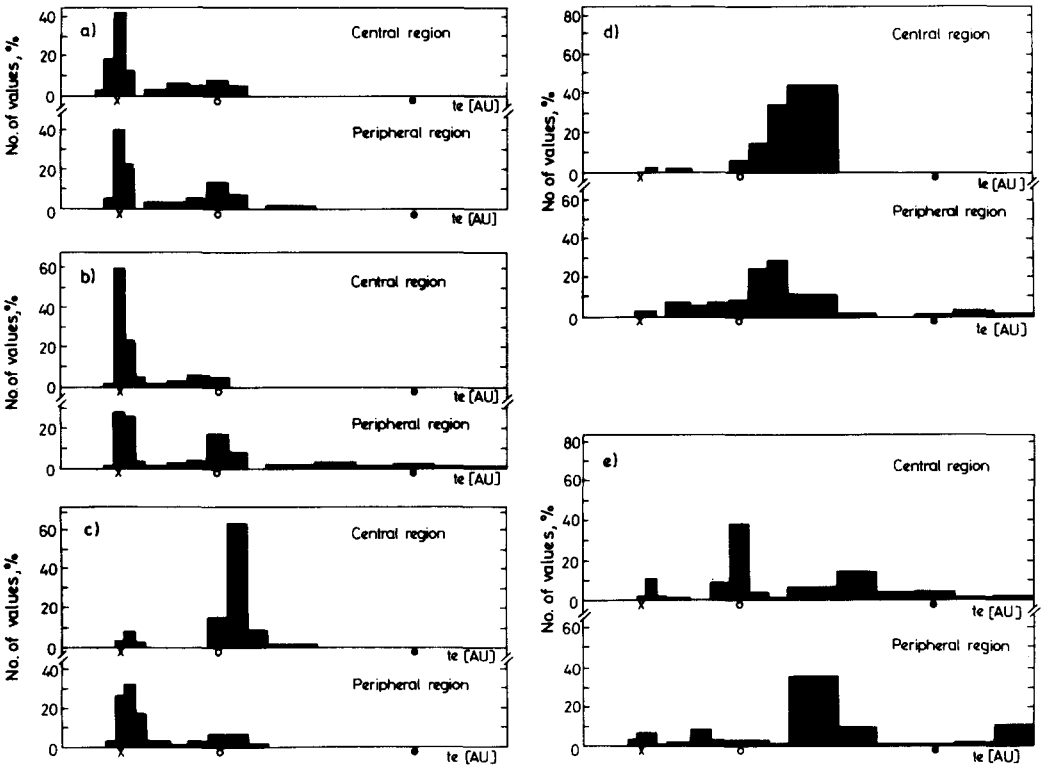

Fig. 2. DNA-histograms from biopsy specimens from a central and a peripheral region in 5 bladder carcinomas.

× *diploid*
○ *tetraploid* } *DNA-value.*
● *octoploid*

peripheral biopsy were used in the sampled material of 123 patients (Tables 2–5 and Fig. 1). In 3 patients biopsies were also taken from distant metastases (Fig. 3).

A control group consisted of 32 specimens of macroscopically normal bladder mucosa. These were collected from patients with benign disorders of the lower urinary tract (mostly benign prostatic hyperplasia) or were taken immediately after death from patients who died without urological disorders.

The age distribution in the TCC group and the control group was similar.

In 20 patients the bladder carcinoma was

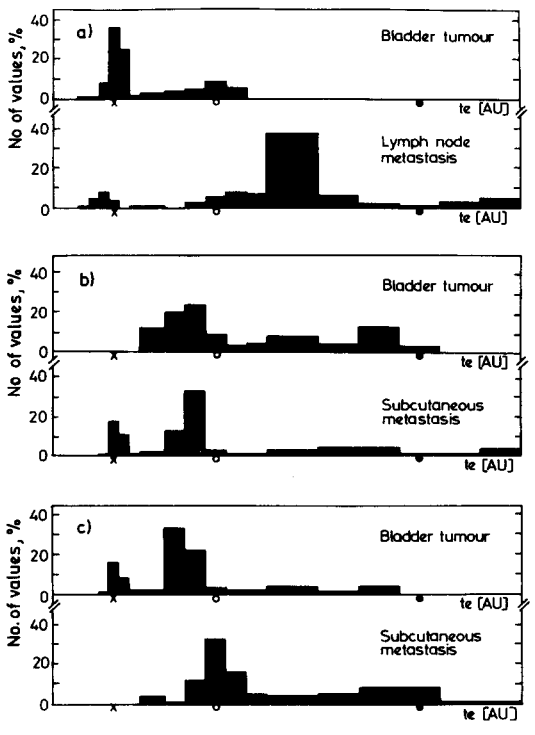

Fig. 3. DNA-histograms from biopsy specimens from patients with bladder carcinoma and distant metastases.
× *diploid*
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when the actual biopsy was taken, regardless of previous surgical intervention. As it was often impossible to distinguish between stage II and stage III in previously operated patients these stages were grouped together. This stage II–III was defined by the presence of a firm mobile induration of any size in the bladder wall, or of a tumour with histologically proven infiltration of the muscular layer of the bladder wall.

Chest X-rays were performed in all patients. Pedal lymphography was done in 29 patients. Only patients with unquestionable evidence of disseminated disease were considered to have metastases.

Table 1 shows the treatment of the patients, following the actual biopsy. Preparation, fixation, staining and mounting of the imprints were done as described previously [6].

The total extinction (*te*) of a Feulgen-stained cell nucleus, here defined as Feulgen DNA-value, was measured by means of a scanning microscope photometer SMP05 (Zeiss, Oberkochen, W. Germany) [6].

A total of 100 round or elipsoid cell nuclei with reticular chromatin pattern was measured in randomly selected regions of the imprints. The mean DNA-value of 30 polymorphonuclear leukocytes in each imprint was used as a reference to the actual specimen's diploid DNA-value (internal standard) [3].

Table 1. Treatment in 123 patients with bladder carcinoma

Clinical stage	Treatment—number of patients					
	Radical surgery	Non-radical surgery	Radical surgery + local chemo-therapy	Non-radical surgery + external irradiation	General chemo-therapy	Total
Stage I	47		14		2	63
Stage II–III	2	5		17	2	26
Stage IV		1		27	6	34

Local chemotherapy: 30 mg Thiotepa intravesically for 30 min after transurethral resection.
External irradiation: 60 Gy/6 weeks to the pelvis by a betatron 33 mV.
General chemotherapy: 5-fluorouracil intravenously 12 mg/kg/week.

verified histologically by the actual biopsy, while 103 patients were admitted with tumour recurrence after surgery in other hospitals. In these patients information was not available to determine the clinical stage of the bladder carcinoma when the initial diagnosis was made. The TNM-system, used for staging of the bladder tumour [5] in all patients, was therefore slightly adjusted in the present study: The terms, stages I–IV, were here used to describe the clinical extent of the bladder tumour at the time

The frequency distribution of 100 Feulgen DNA-values in each specimen (DNA-histogram) was drawn using the following definitions:

The diploid region was defined as the diploid DNA-value ± 25%. The tetraploid and octoploid regions were 2-fold and 4-fold the diploid DNA-value respectively, ± 25%. The diploid, tetraploid and octoploid regions were each

Table 2. Number of diploid and non-diploid bladder carcinomas related to histological grade and clinical stage

Histological grade	Grade I			Grade II			Grade III			Grade IV			Total		
DNA-steml. ploidy	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All
Clinical stage:															
Stage I	26	2	28	13	8	21	4	10	14				43	20	63
Stage II–III	2		2	1	2	3	5	14	19		2	2	8	18	26
Stage IV	2		2	3	4	7	2	15	17	5	3	8	12	22	34
Total	30	2	32	17	14	31	11	39	50	5	5	10	63	60	123

Table 3. Mean percentage of DNA-stemline values in bladder carcinomas with diploid and non-diploid DNA-stemlines related to histological grade and clinical stage

Histological grade	Grade I			Grade II			Grade III			Grade IV			Total		
	No. of values (%)			No. of values (%)			No. of values (%)			No. of values (%)			No. of values (%)		
DNA-steml. ploidy	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All
Clinical stage:															
Stage I	88	61	86	82	74	79	74	69	70				85	70	80
Stage II–III	80		80	78	48	58	63	63	63		49	49	69	60	63
Stage IV	81		81	71	63	66	65	61	61	60	53	57	67	60	62
Total	87	61	85	80	67	74	67	64	64	60	51	55	80	63	72

subdivided into 5 intervals, each comprising 10% of the diploid, tetraploid and octoploid figure respectively. The definition of a DNA-stemline has been described elsewhere [6]. DNA-values within the DNA-stemline region were called DNA-stemline values. The mean of the DNA-stemline values yielded the modal DNA-value [4].

Following imprint preparation the tissue fragments were fixed in formalin and embedded in paraffin.

Grading of the histological haematoxylin-eosin-stained sections was done according to Mostofi [7] denominating all epithelial bladder tumours with cellular anaplasia as carcinomas, regardless whether infiltrative growth was demonstrated or not.

Statistical evaluation

The actuarial survival of patients was calculated from the date of the actual biopsy using the life table method [8]. Differences between survival rates at a definite point of time were tested by the Student's *t* test.

Differences between survival curves for different groups of patients were also tested by the logrank test [9]. The original test is not directly applicable for data grouped in time. The scheme was therefore modified making all possible rankings within each group equally probable when evaluating the estimator (e) for the logarithm of the survival curve. The numbers for death expectancy E_j obtained by cumulating the e-values for the j'th group of patients, were compared to the number of deaths 0_j by the χ^2 test.

RESULTS

In each of the control specimens a diploid DNA-stemline was found. The control group comprised, on the average, 2.4% tetraploid and 0.1% octoploid DNA-values. In 6 individual control specimens a frequency of 8–10% polyploid DNA-values was found.

Table 2 shows the distribution of DNA-stemlines in relation to histological grade and clinical stage. Sixty-three specimens showed a diploid DNA-stemline, while in 60 the stemline was non-diploid. Of the latter, 35 were tetraploid and 6 showed bimodality (diploid and tetraploid DNA-stemline).

The relative frequency of non-diploid tumours increased with decreasing histological differentiation and increasing clinical stage.

In Table 3 the mean percentage of DNA-stemline values is shown for diploid and non-diploid tumours. Within the same histological grade the number of DNA-stemline values tended to decrease with increasing clinical stage, most clearly demonstrated when comparing stage I with more advanced tumours. Especially in diploid tumours increasing clinical stage and decreasing histological differentiation were correlated with low numbers of DNA-stemline values. Non-diploid tumours showed no such clear correlation, but the DNA-stemlines in non-diploid tumours generally comprised less DNA-stemline values than observed in diploid tumours.

Table 4 shows the relationship between the DNA-stemline ploidy and the number of patients with obvious metastases found either at the time of the actual biopsy or during follow-up. Metastases were twice as often associated with non-diploid tumours as with diploid carcinomas. A particularly high frequency of metastases was noted in non-diploid stage IV tumours.

The actuarial survival of patients with diploid TCC was compared to that of patients with non-diploid tumours (Fig. 1a). After 24 months more patients with diploid tumours than with non-diploid tumours were alive ($P<0.05$). The logrank test showed a statistically significant difference between the survival curves. One obvious reason for this is that 43 of 63 diploid carcinomas were at stage I. Therefore, in Fig. 1(b) and 1(c) the survival curves were given separately for patients with stage I disease and with advanced stages (stages II–III and stage IV). Patients with advanced diploid tumours had a higher cumulative survival rate at 18 months than those with non-diploid tumours

Table 4. *Correlation between clinical stage and tumour's DNA-stemline ploidy in* 27 *patients with metastasizing bladder carcinoma*

Clinical stage		Stage I	Stage II–III	Stage IV	Total
Diploid tumours	Number of patients	3(43)	2(8)	4(12)	9(63)
Non-diploid tumours	Number of patients	3(20)	2(18)	13(22)	18(60)

Figures in brackets refer to the total number of patients within each subgroup.

Table 5. Number of patients with poorly differentiated and undifferentiated locally advanced bladder carcinoma surviving 18 months related to the tumour's DNA-stemline ploidy

DNA-stemline ploidy	Number of patients		
	Total	Alive	Dead
Diploid	10	6	4
Non-diploid	30	10	20
Total	40	16	24

($P \simeq 0.10$). After 24 months the survival rate was the same for patients with diploid and non-diploid advanced bladder tumours. No significant difference was observed in the survival regarding patients with stage I disease.

Table 5 shows the number of patients with poorly differentiated and undifferentiated locally advanced bladder carcinoma (grade III and grade IV, stages II–III and stage IV) surviving 18 months. Patients with diploid tumours seem to do better than those with non-diploid tumours.

In 3 of the 5 patients with two biopsy specimens from the same tumour, different modal DNA-values could be demonstrated in the central and peripheral tumour area, demonstrated both in stage I tumours (Figs. 2c and 2d) and in a stages II–III tumour (Fig. 2e).

Comparison of the DNA-histogram from a bladder tumour with that of a distant metastasis was made in each of 3 patients (Fig. 3). The metastases in these patients had non-diploid DNA-stemlines, even in the patient with a diploid bladder tumour (Fig. 3a).

DISCUSSION

We have based our control group on specimens taken from patients with benign urological disease or from deceased individuals without urological disease. As the bladder mucosa often shows inflammatory reactions in patients with urological disease it was necessary to accept the presence of a slight degree of inflammation in some of the first-mentioned controls.

We found a low mean percentage (2.4%) of tetraploid DNA-values in our control group while some individual control specimens showed between 8 and 10% polyploid cell nuclei.

Levi *et al.* [10] found about 25% polyploid cell nuclei in human urothelium. Tribukait *et al.* [11] and Freni *et al.* [12] examined bladder washings and urine from patients with benign urothelial disorders by flow-cytometry. They found an almost pure diploid DNA-stemline with only few polyploid DNA-values. Freni *et al.* [12] observed about 10% non-diploid DNA-values in urothelial cells obtained by scraping normal bladder mucosa.

Some of the discrepancies found in the frequency of polyploid cells in the bladder mucosa in different studies may be explained by different techniques in obtaining the specimens and by different methods in selection of cell nuclei to be measured.

Based on our own studies and Freni's *et al.* [12] observations we assume that normal urothelium does not contain more than 15% non-diploid DNA-values, most of them in the tetraploid range.

Sixty-three bladder carcinomas were characterized as diploid and 60 as non-diploid. These results were based on DNA-measurements in a single tumour biopsy. However, intratumoural variations of the DNA-stemline ploidy may occur (Fig. 2). This limitation of the method, used in this study, should be regarded when evaluating the present results.

In this study the cytophotometric results were correlated with clinical stage and histological grade of the bladder tumour, though the clinical stage according to the TNM-system [5] does not always describe the real extent of the disease [13]. However, no other clinical classification system is available which can easily be used in all patients with bladder carcinoma.

The preponderance of non-diploid tumours found in clinically advanced and histologically dedifferentiated carcinomas is in agreement with the results of others [10, 14–17]. We found, however, that 5 out of 10 undifferentiated bladder tumours had diploid DNA-stemlines. Contrary to what is observed in well differentiated tumours, these undifferentiated diploid bladder carcinomas had "weak" DNA-stemlines with low numbers of DNA-stemline values.

The number of DNA-values outside the DNA-stemline increased with decreasing histological differentiation. In dedifferentiated bladder tumours an increased uptake of ^{3}H-thymidine has been observed as compared to well differentiated carcinomas [18, 19]. The raised numbers of non-DNA-stemline values may therefore indicate an increased growth fraction in dedifferentiated tumours as compared to well differentiated ones. In addition, the increased frequency of non-DNA-stemline values in dedifferentiated bladder carcinomas may also reflect that such tumours have lost the relative homogeneity of the normal urothelium and that aneuploid and polyploid cell clones are developing as a result of chromosomal changes.

In the 3 patients with metastasizing bladder

carcinoma a weak DNA-stemline and numerous non-diploid cells were observed in the primary tumour. Non-diploid tumour cells may have selective advantages leading to the predominance of these cells in metastases, even in those from diploid bladder tumours. The observation that DNA-stemlines in metastases may show a higher degree of ploidization than those in the corresponding primary tumours, has also been made by Avtandilov *et al.* [20] and Rabotti [21].

From the literature, only limited information is available about the prognostic significance of cytophotometric DNA-measurements in human bladder carcinoma [22].

In the present study the survival of patients was calculated from the date when the actual biopsy was taken, as only at this time comparable results of the clinical, histological and cytophotometric examination could be obtained,

In our series of 123 patients those with diploid carcinomas had a higher survival rate than those with non-diploid tumours (Fig. 1a). However, the prognostic significance of cytophotometric DNA-studies can only be proven when comparing patients with the same clinical stage and with the same histological grade. In this study the prognostic value of DNA-measurements was indicated by the observation that patients with diploid locally advanced dedifferentiated tumours had a better prognosis than those with non-diploid carcinomas.

Clinically, non-diploid bladder carcinomas seemed to be more aggressive than diploid tumours, by more extensive local growth, by metastasizing more frequently and by leading to the patient's death within a shorter period of time.

Another possible explanation of the observed differences between the survival curves might be that diploid bladder carcinomas corresponded differently to treatment than non-diploid ones. Of 16 patients with advanced diploid bladder tumours treated by non-radical surgery and radiotherapy, 6 were without tumour after 18 months whereas only 4 of 26 likewisely treated patients with non-diploid carcinomas were tumour-free.

Whatever the explanation may be for the better survival of patients with diploid bladder carcinomas the present study indicates that cytophotometric DNA-measurements have clinical and histopathological significance which may be valuable in determining the prognosis in patients with TCC.

Cytophotometric DNA-studies in imprints from solid tumours are time-consuming. Only a limited number of cell nuclei can be evaluated in each specimen. More rapid DNA-determination by pulse-cytophotometry may therefore offer some advantages, but this method's role in studies of human bladder carcinoma needs further investigation [11, 12].

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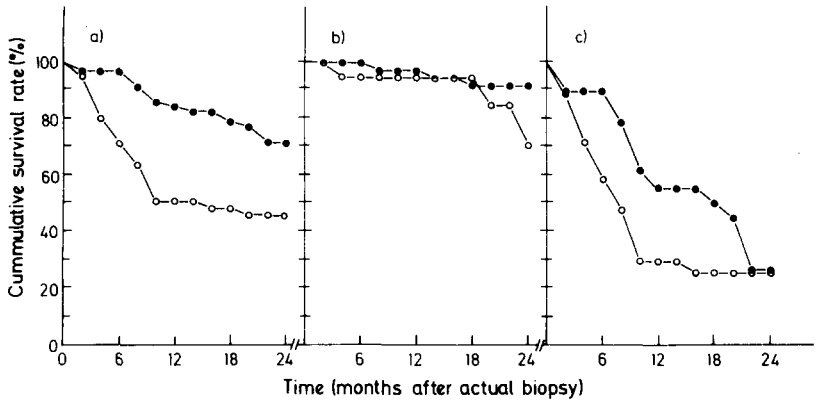

Fig. 1. Actuarial survival in patients with bladder carcinoma calculated from the actual biopsy. Tumours with diploid DNA-stemlines (● ● ●). Tumours with non-diploid DNA-stemlines (○ ○ ○). (a) All tumours (63 diploid/60 non-diploid) (b) Stage I tumours (43 diploid/20 non-diploid) (c) Stage II–III tumours (8 diploid/18 non-diploid) and Stage IV tumours (12 diploid/22 non-diploid).

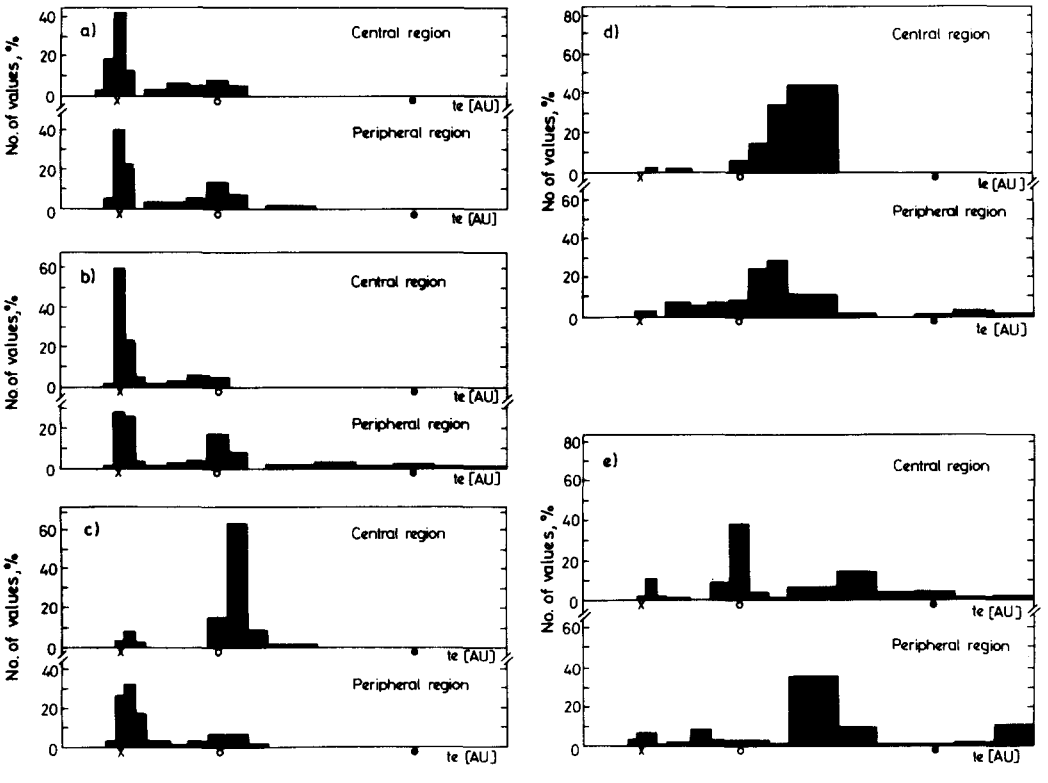

Fig. 2. DNA-histograms from biopsy specimens from a central and a peripheral region in 5 bladder carcinomas.

× *diploid*
○ *tetraploid* } *DNA-value.*
● *octoploid*

peripheral biopsy were used in the sampled material of 123 patients (Tables 2–5 and Fig. 1). In 3 patients biopsies were also taken from distant metastases (Fig. 3).

A control group consisted of 32 specimens of macroscopically normal bladder mucosa. These were collected from patients with benign disorders of the lower urinary tract (mostly benign prostatic hyperplasia) or were taken immediately after death from patients who died without urological disorders.

The age distribution in the TCC group and the control group was similar.

In 20 patients the bladder carcinoma was

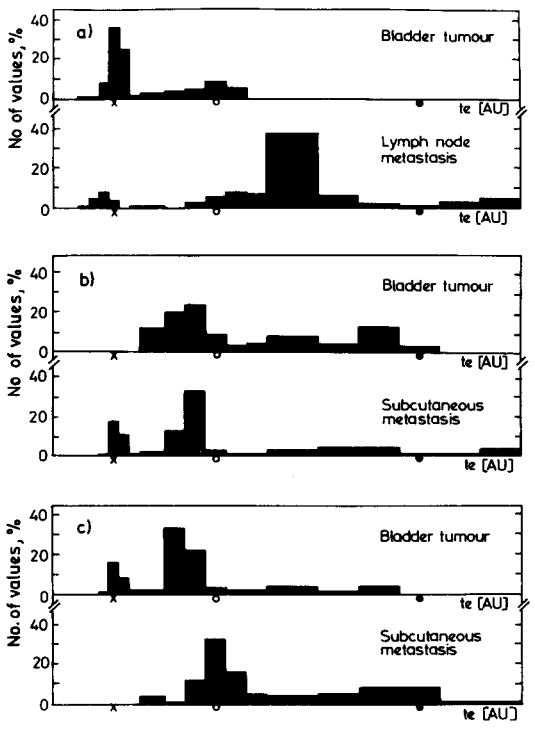

Fig. 3. DNA-histograms from biopsy specimens from patients with bladder carcinoma and distant metastases.
× *diploid*
○ *tetraploid* } *DNA-value.*
● *octoploid*

when the actual biopsy was taken, regardless of previous surgical intervention. As it was often impossible to distinguish between stage II and stage III in previously operated patients these stages were grouped together. This stage II–III was defined by the presence of a firm mobile induration of any size in the bladder wall, or of a tumour with histologically proven infiltration of the muscular layer of the bladder wall.

Chest X-rays were performed in all patients. Pedal lymphography was done in 29 patients. Only patients with unquestionable evidence of disseminated disease were considered to have metastases.

Table 1 shows the treatment of the patients, following the actual biopsy. Preparation, fixation, staining and mounting of the imprints were done as described previously [6].

The total extinction (*te*) of a Feulgen-stained cell nucleus, here defined as Feulgen DNA-value, was measured by means of a scanning microscope photometer SMP05 (Zeiss, Oberkochen, W. Germany) [6].

A total of 100 round or elipsoid cell nuclei with reticular chromatin pattern was measured in randomly selected regions of the imprints. The mean DNA-value of 30 polymorphonuclear leukocytes in each imprint was used as a reference to the actual specimen's diploid DNA-value (internal standard) [3].

Table 1. Treatment in 123 patients with bladder carcinoma

Clinical stage	Treatment—number of patients					
	Radical surgery	Non-radical surgery	Radical surgery + local chemo-therapy	Non-radical surgery + external irradiation	General chemo-therapy	Total
Stage I	47		14		2	63
Stage II–III	2	5		17	2	26
Stage IV		1		27	6	34

Local chemotherapy: 30 mg Thiotepa intravesically for 30 min after transurethral resection.
External irradiation: 60 Gy/6 weeks to the pelvis by a betatron 33 mV.
General chemotherapy: 5-fluorouracil intravenously 12 mg/kg/week.

verified histologically by the actual biopsy, while 103 patients were admitted with tumour recurrence after surgery in other hospitals. In these patients information was not available to determine the clinical stage of the bladder carcinoma when the initial diagnosis was made. The TNM-system, used for staging of the bladder tumour [5] in all patients, was therefore slightly adjusted in the present study: The terms, stages I–IV, were here used to describe the clinical extent of the bladder tumour at the time

The frequency distribution of 100 Feulgen DNA-values in each specimen (DNA-histogram) was drawn using the following definitions:

The diploid region was defined as the diploid DNA-value ± 25%. The tetraploid and octoploid regions were 2-fold and 4-fold the diploid DNA-value respectively, ± 25%. The diploid, tetraploid and octoploid regions were each

Table 2. Number of diploid and non-diploid bladder carcinomas related to histological grade and clinical stage

Histological grade	Grade I			Grade II			Grade III			Grade IV			Total		
DNA-steml. ploidy	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All
Clinical stage:															
Stage I	26	2	28	13	8	21	4	10	14				43	20	63
Stage II–III	2		2	1	2	3	5	14	19		2	2	8	18	26
Stage IV	2		2	3	4	7	2	15	17	5	3	8	12	22	34
Total	30	2	32	17	14	31	11	39	50	5	5	10	63	60	123

Table 3. Mean percentage of DNA-stemline values in bladder carcinomas with diploid and non-diploid DNA-stemlines related to histological grade and clinical stage

Histological grade	Grade I			Grade II			Grade III			Grade IV			Total		
	No. of values (%)			No. of values (%)			No. of values (%)			No. of values (%)			No. of values (%)		
DNA-steml. ploidy	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All	Dipl.	Non-dipl.	All
Clinical stage:															
Stage I	88	61	86	82	74	79	74	69	70				85	70	80
Stage II–III	80		80	78	48	58	63	63	63		49	49	69	60	63
Stage IV	81		81	71	63	66	65	61	61	60	53	57	67	60	62
Total	87	61	85	80	67	74	67	64	64	60	51	55	80	63	72

subdivided into 5 intervals, each comprising 10% of the diploid, tetraploid and octoploid figure respectively. The definition of a DNA-stemline has been described elsewhere [6]. DNA-values within the DNA-stemline region were called DNA-stemline values. The mean of the DNA-stemline values yielded the modal DNA-value [4].

Following imprint preparation the tissue fragments were fixed in formalin and embedded in paraffin.

Grading of the histological haematoxylin-eosin-stained sections was done according to Mostofi [7] denominating all epithelial bladder tumours with cellular anaplasia as carcinomas, regardless whether infiltrative growth was demonstrated or not.

Statistical evaluation

The actuarial survival of patients was calculated from the date of the actual biopsy using the life table method [8]. Differences between survival rates at a definite point of time were tested by the Student's *t* test.

Differences between survival curves for different groups of patients were also tested by the logrank test [9]. The original test is not directly applicable for data grouped in time. The scheme was therefore modified making all possible rankings within each group equally probable when evaluating the estimator (e) for the logarithm of the survival curve. The numbers for death expectancy E_j obtained by cumulating the e-values for the j'th group of patients, were compared to the number of deaths 0_j by the χ^2 test.

RESULTS

In each of the control specimens a diploid DNA-stemline was found. The control group comprised, on the average, 2.4% tetraploid and 0.1% octoploid DNA-values. In 6 individual control specimens a frequency of 8–10% polyploid DNA-values was found.

Table 2 shows the distribution of DNA-stemlines in relation to histological grade and clinical stage. Sixty-three specimens showed a diploid DNA-stemline, while in 60 the stemline was non-diploid. Of the latter, 35 were tetraploid and 6 showed bimodality (diploid and tetraploid DNA-stemline).

The relative frequency of non-diploid tumours increased with decreasing histological differentiation and increasing clinical stage.

In Table 3 the mean percentage of DNA-stemline values is shown for diploid and non-diploid tumours. Within the same histological grade the number of DNA-stemline values tended to decrease with increasing clinical stage, most clearly demonstrated when comparing stage I with more advanced tumours. Especially in diploid tumours increasing clinical stage and decreasing histological differentiation were correlated with low numbers of DNA-stemline values. Non-diploid tumours showed no such clear correlation, but the DNA-stemlines in non-diploid tumours generally comprised less DNA-stemline values than observed in diploid tumours.

Table 4 shows the relationship between the DNA-stemline ploidy and the number of patients with obvious metastases found either at the time of the actual biopsy or during follow-up. Metastases were twice as often associated with non-diploid tumours as with diploid carcinomas. A particularly high frequency of metastases was noted in non-diploid stage IV tumours.

The actuarial survival of patients with diploid TCC was compared to that of patients with non-diploid tumours (Fig. 1a). After 24 months more patients with diploid tumours than with non-diploid tumours were alive ($P<0.05$). The logrank test showed a statistically significant difference between the survival curves. One obvious reason for this is that 43 of 63 diploid carcinomas were at stage I. Therefore, in Fig. 1(b) and 1(c) the survival curves were given separately for patients with stage I disease and with advanced stages (stages II–III and stage IV). Patients with advanced diploid tumours had a higher cumulative survival rate at 18 months than those with non-diploid tumours

Table 4. *Correlation between clinical stage and tumour's DNA-stemline ploidy in* 27 *patients with metastasizing bladder carcinoma*

Clinical stage		Stage I	Stage II–III	Stage IV	Total
Diploid tumours	Number of patients	3(43)	2(8)	4(12)	9(63)
Non-diploid tumours	Number of patients	3(20)	2(18)	13(22)	18(60)

Figures in brackets refer to the total number of patients within each subgroup.

Table 5. Number of patients with poorly differentiated and undifferentiated locally advanced bladder carcinoma surviving 18 months related to the tumour's DNA-stemline ploidy

DNA-stemline ploidy	Number of patients		
	Total	Alive	Dead
Diploid	10	6	4
Non-diploid	30	10	20
Total	40	16	24

($P \simeq 0.10$). After 24 months the survival rate was the same for patients with diploid and non-diploid advanced bladder tumours. No significant difference was observed in the survival regarding patients with stage I disease.

Table 5 shows the number of patients with poorly differentiated and undifferentiated locally advanced bladder carcinoma (grade III and grade IV, stages II–III and stage IV) surviving 18 months. Patients with diploid tumours seem to do better than those with non-diploid tumours.

In 3 of the 5 patients with two biopsy specimens from the same tumour, different modal DNA-values could be demonstrated in the central and peripheral tumour area, demonstrated both in stage I tumours (Figs. 2c and 2d) and in a stages II–III tumour (Fig. 2e).

Comparison of the DNA-histogram from a bladder tumour with that of a distant metastasis was made in each of 3 patients (Fig. 3). The metastases in these patients had non-diploid DNA-stemlines, even in the patient with a diploid bladder tumour (Fig. 3a).

DISCUSSION

We have based our control group on specimens taken from patients with benign urological disease or from deceased individuals without urological disease. As the bladder mucosa often shows inflammatory reactions in patients with urological disease it was necessary to accept the presence of a slight degree of inflammation in some of the first-mentioned controls.

We found a low mean percentage (2.4%) of tetraploid DNA-values in our control group while some individual control specimens showed between 8 and 10% polyploid cell nuclei.

Levi *et al.* [10] found about 25% polyploid cell nuclei in human urothelium. Tribukait *et al.* [11] and Freni *et al.* [12] examined bladder washings and urine from patients with benign urothelial disorders by flow-cytometry. They found an almost pure diploid DNA-stemline with only few polyploid DNA-values. Freni *et al.* [12] observed about 10% non-diploid DNA-values in urothelial cells obtained by scraping normal bladder mucosa.

Some of the discrepancies found in the frequency of polyploid cells in the bladder mucosa in different studies may be explained by different techniques in obtaining the specimens and by different methods in selection of cell nuclei to be measured.

Based on our own studies and Freni's *et al.* [12] observations we assume that normal urothelium does not contain more than 15% non-diploid DNA-values, most of them in the tetraploid range.

Sixty-three bladder carcinomas were characterized as diploid and 60 as non-diploid. These results were based on DNA-measurements in a single tumour biopsy. However, intratumoural variations of the DNA-stemline ploidy may occur (Fig. 2). This limitation of the method, used in this study, should be regarded when evaluating the present results.

In this study the cytophotometric results were correlated with clinical stage and histological grade of the bladder tumour, though the clinical stage according to the TNM-system [5] does not always describe the real extent of the disease [13]. However, no other clinical classification system is available which can easily be used in all patients with bladder carcinoma.

The preponderance of non-diploid tumours found in clinically advanced and histologically dedifferentiated carcinomas is in agreement with the results of others [10, 14–17]. We found, however, that 5 out of 10 undifferentiated bladder tumours had diploid DNA-stemlines. Contrary to what is observed in well differentiated tumours, these undifferentiated diploid bladder carcinomas had "weak" DNA-stemlines with low numbers of DNA-stemline values.

The number of DNA-values outside the DNA-stemline increased with decreasing histological differentiation. In dedifferentiated bladder tumours an increased uptake of ^{3}H-thymidine has been observed as compared to well differentiated carcinomas [18, 19]. The raised numbers of non-DNA-stemline values may therefore indicate an increased growth fraction in dedifferentiated tumours as compared to well differentiated ones. In addition, the increased frequency of non-DNA-stemline values in dedifferentiated bladder carcinomas may also reflect that such tumours have lost the relative homogeneity of the normal urothelium and that aneuploid and polyploid cell clones are developing as a result of chromosomal changes.

In the 3 patients with metastasizing bladder

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Tumour Regression and Survival of Patients with Disseminated Malignant Melanoma Treated with Chemotherapy and Specific Active Immunotherapy*

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Abstract—*We have treated 59 patients with disseminated or inoperable malignant melanoma with a combination of chemotherapy (D.T.I.C.) and immunotherapy (comprising irradiated allogeneic melanoma cells plus BCG) and have followed their progress for a minimum of ten months.*

Objective tumour regression was observed in 44% of the patients but most of these patients relapsed despite continued treatment, the longest complete regression lasting 21 months. Patients with disease confined to the skin, lymph nodes or lungs had a higher regression rate (19/34 as against 7/25) and survived longer than those with visceral, osseous or cerebral metastases. Complete regressions were commoner in women but the overall regression rate was the same in both sexes.

By comparison of these results with literature controls and from the inexorable decline in the survival curves we conclude that despite its apparent effect on objective regression rate this form of combination immunotherapy–chemotherapy has little or no worthwhile effect on survival.

INTRODUCTION

THE OVERALL results of cytotoxic chemotherapy in the treatment of the "final common pathway" of malignant melanoma are disappointing. At present DTIC (5, 3, 3-dimethyl-1-triazeno imidazole 4-carboxamide) is the most active single agent providing objective regression in about 20% of treated patients [1] but with little or no effect on survival. Drug combinations, some including DTIC, have so far proved to be disappointing since they produce more side effects than DTIC alone but no significant increase in overall regression rate [1–4]. Immunotherapy is being evaluated in many centres [5], but so far is of little or no proven value. Our own studies have indicated [6–8] that immunization with irradiated melanoma cells admixed with BCG can lead to predictable changes in some measurable parameters of host responses to malignant melanoma, i.e., can evoke the appearance of tumour-directed cytotoxic lymphocytes and the disappearance of inhibitory factors from the serum. This form of immunization was tested in a group of patients receiving cytotoxic chemotherapy in an uncontrolled study and we noted an objective regression rate higher than that anticipated from historical and literature controls [8].

We have extended these observations and here report a study of 59 patients which indicates that, while the objective regression rate remains high, there appears to be no significant effect on survival and that some patients relapse while on treatment. We also describe several features of the patients which seem to have prognostic significance.

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MATERIAL AND METHODS

Patients

Between May 1973 and October 1975 a total

of 66 patients referred to the Royal Marsden Hospital, Sutton, with disseminated or inoperable malignant melanoma were treated with the combined chemo-immunotherapy protocol. Seven started this treatment at a very advanced stage of their disease and either died before completing the first course or were too ill to attend for further treatment. The remaining 59 patients were treated for a minimum of 6 weeks, none were withdrawn before 6 weeks since regression often occurred after 2 or 3 courses. There were 26 men with a mean age of 45.9 and 33 women with a mean age of 48.2 yr. Patients varied considerably in the amount of tumour present and the sites of overt dissemination at the start of medical treatment, but could be divided into two broad groups: those with disease confined to skin, lymph nodes or lungs (34 cases—lung disease was included in this group since it was our earlier experience that disease confined to the lungs regressed as frequently as nodal or cutaneous metastases) and those with visceral, cerebral or bone metastases (25 cases), since prognosis is known to be particularly bad in the latter group [4]. All patients have been followed up to death or for a minimum of 10 months, and we present data on the regression rate, duration of regression and survival from the start of chemo-immunotherapy. Since this was an extended pilot study there were no concurrent control patients, the shape of the survival curve leading us to abandon a formal controlled trial. Our regression and survival data were compared to those obtained in recently published studies from other centres [2, 4]. Furthermore our own historical survival data are similar to those reported by Einhorn and colleages [2].

Treatment Protocol

(a) *Chemotherapy.* All patients received DTIC every 4 weeks. Initially the dose used was 2.5 mg/kg intravenously daily for five consecutive days combined with Vincristine 1.4 mg/m^2 intravenously on the first day. Because of some cases of neurotoxicity and lack of evidence of any clear cut benefit from the addition of vincristine [9] this agent was not used in the last 27 patients and the dose of DTIC was increased to 250 mg/m^2 for 5 days. There were few serious adverse reactions and no deaths attributed to the DTIC, although vomiting for a few hr after the injection was common. One patient developed thrombocytopenic purpura on DTIC 250 mg/m^2 but tolerated a reduced dose.

(b) *Immunotherapy.* This consisted of 2×10^7 irradiated allogeneic malignant melanoma cells plus 50 μg of percutaneous Glaxo BCG by intradermal injection as previously described [8]. This "immunotherapy" was given midway between chemotherapy courses.

Alternating chemotherapy and immunotherapy was continued either until it was obvious that the disease was unresponsive or until the patient had been in complete remission for at least 6 months.

RESULTS

The clinical effect of treatment was judged to be (a) no response; (b) partial regression or (c) complete regression. Partial regression is defined as a decrease in the mean diameter of measurable lesions of 50% for at least 30 days in the absence of disease progression elsewhere or the presence of static disease elsewhere. Patients with less than 50% regression or whose disease remained static were included amongst the non-responders. Furthermore, patients who showed more than 50% regression of some lesions while other lesions were progressing were regarded as non-responders. Using these criteria 33 (56%) of the 59 evaluable cases did not respond to treatment. Of the 25 responders 6 went into complete regression and 20 had partial regressions (i.e., overall regression rate = 44%).

Regression rate according to extent of metastases

Although the overall response rate (of evaluable cases) in males and females was similar (males 11/26, females 15/33) only 1 male patient achieved complete regression compared to 5 females. This difference was not related to the extent of the disease since regression of visceral and even bone metastases were seen in the women whereas many men with skin and nodal involvement did not achieve complete regression.

Regression rate according to extent of metastases

In patients with disease confined to skin, lymph nodes or lungs, the regression rate was 56% (19/34). This is exactly twice that of patients with visceral disease, 28% (7/25), in whom there was only one complete regression. Table 1 lists the sites of overt metastasis at the start of chemo-immunotherapy and the number of patients in whom disease regression took place. In several patients disease regressed at some sites while remaining static at others. Table 2 summarises the regression rates according to sex and the presence or absence of visceral metastases.

Table 1. Sites of metastasis at the start of chemoimmunotherapy and the number of patients in whom regression was seen

Site	Number of patients with involvement	Number of patients with disease regression
Skin	37	18 (49%)
Lymph nodes	27	14 (52%)
Lungs	21	9 (43%)
Bone	10	2 (20%)
Liver	16	5 (31%)
Brain	6	1
Bowel	3	0
Nasal mucosa	1	0
Salivary gland	2	1
Adrenal	1	0

Table 2. Regression rates according to sex and the presence or absence of visceral metastases

	All sites	Visceral disease	Disease confined to skin, lymph nodes or lungs
Men and women	26/59 (44%)	7/25 (28%)	19/34 (56%)
Men	11/26	3/12	8/14
Women	15/33	4/13	11/20

Regression rate according to duration of history

The interval between primary excision and the start of chemo-immunotherapy for surgically incurable disease ranged from 2 to 300 months, the median being 47 months in patients who showed disease regression and 28 months in those who did not. A test for the mean (log) duration of history showed no significant difference—$t=0.72$, degrees of freedom (d.f.) 53, $P<0.5$.

Regression rate according to age

The mean age of all patients was 47 yr, of regressors 54 yr and of non-regressors 42 yr. These results were tested by Chi square analysis which showed that the difference was significant

$$(\chi^2=9.6, \text{d.f.}=3, P<0.05)$$

This difference in mean age between regressors and non-regressors was almost exactly the same in the men as in the women.

Interval from start of treatment to start of regression

All but two regressions had become clinically detectable by the third month of treatment, 9 of these occurring within the first 4 weeks. There was no significant correlation between the speed with which a regression became evident and its duration, except that the 2 regressions noted after 3 months were only of short duration.

Duration of regression

All patients who achieved complete regression have since relapsed, with a median regression length of 12 months. Three patients relapsed while still on treatment.

The 20 patients achieving partial regression have now all relapsed despite continuing on the full treatment protocol. Partial regressions were sustained for a shorter period (median 3 months) than complete regressions.

In patients with visceral, cerebral or bone disease the median duration of regression was 3 months whereas in those with nodal, pulmonary or cutaneous deposits it was 6.5 months.

Survival from the start of chemo-immunotherapy

Patients showing disease regression lived longer than those who did not (Fig. 1). None of the non-responders survived for more than 9 months from the start of chemo-immunotherapy, whereas 6 out of the 26 remitters are still alive 10, 14, 18, 18, 25 and 28 months after the start of treatment. Figures 2 and 3 divide the patients into those with visceral, cerebral or bone involvement and those with disease confined to skin, lymph nodes or lung. The survival curves for patients with visceral disease show a very poor prognosis which is improved only slightly in those with disease regression. This group includes one complete regression, a patient who had multiple osteolytic bone deposits in addition to lung, skin and regional and distant lymph node involvement. Those patients with metastases confined to skin, lymph node or lung showed longer median survival in those achieving disease regression (15 months) compared to 6 months in non-remitters. The pronounced shoulder in the survival curve

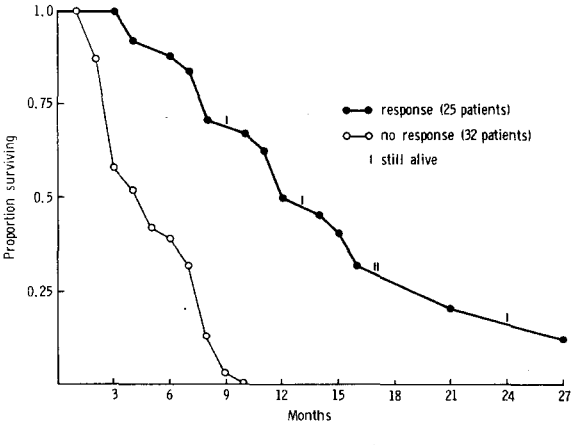

Fig. 1. Survival curves (life table) for all patients.

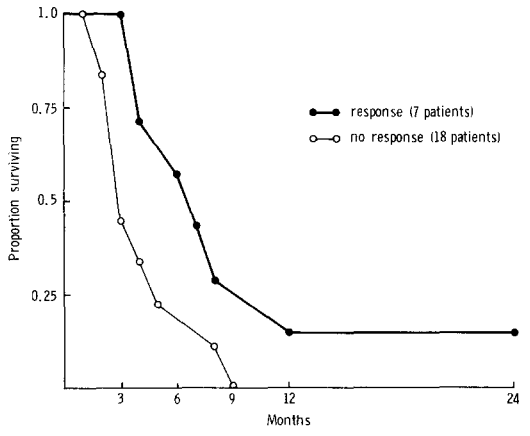

Fig. 2. Survival curves (life tables) for patients with overt visceral, osseous or cerebral metastases.

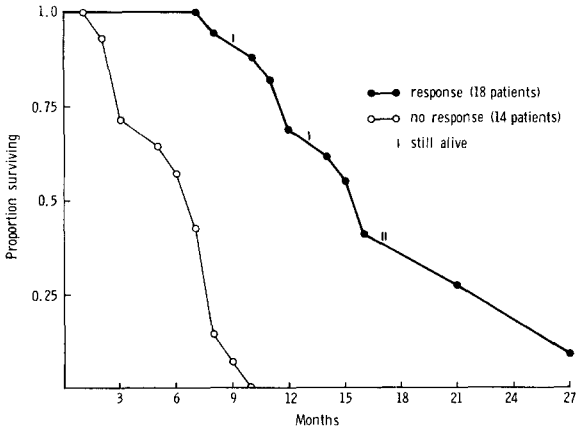

Fig. 3. Survival curves (life table) for patients with disease limited to skin, lymph nodes or lung.

of responders in this group (Fig. 3) is the main difference between its shape and that of all responders (Fig. 1), and this plus the absence of a tail of survivors suggests that the improved survival of patients with disease limited to skin, node or lung may be a function of the disease rather than the treatment, i.e., treatment was started earlier because metastases at these sites are more easily detected.

DISCUSSION

Despite widespread enthusiasm for immunotherapy as a form of cancer treatment, there is little convincing clinical evidence of its effectiveness and the rationale behind many of the forms of treatment in current use remains poorly defined.

This extended pilot study confirms our earlier findings [8] which suggested that specific active immunotherapy increases the overall objective regression rate, but the shape of the survival curves, with the complete absence of any tail, shows that this treatment is incapable of significantly improving survival in patients with advanced disease. The median survival times achieved are very similar to those described by other authors [2, 4] and closely resemble the figures published by De Vita and Fisher [10]. Indeed, all of the patients who had partial objective regressions relapsed while continuing treatment with alternating immunotherapy and chemotherapy. Such results are reminiscent of the effects of unsuccessful cytotoxic chemotherapy in other tumours such as colorectal cancer where objective regressions can be achieved but are not associated with significant prolongation of life.

The overall regression rate of 44% is about twice that achieved in most large series using chemotherapy alone and was obtained with a relatively non-toxic regime. Although regressions were seen most frequently in patients with disease confined to the skin, lymph nodes or the lungs, we do not believe that the selection of patients alone can account for the high regression rate since we obtained a rate of 19/34 (56%) in this group and 7/25 (28%) in those with visceral, cerebral and bone metastases, both figures being substantially higher than those reported for the effects of chemotherapy alone in such patients [1, 2].

Newlands and his colleagues [11] have reported a study in which specific active immunotherapy combined with chemotherapy was compared with chemotherapy alone in similar patients to our own. Their results clearly demonstrated the absence of any effect on survival despite showing an effect of immunotherapy on the regression rate, which was 4 out of 17 (23%) in patients receiving chemotherapy alone and 9 out of 19 (47%) in patients receiving chemotherapy plus immunotherapy.

In our hands the combined treatment induced 56% regressions in patients with skin, lymph node and lung disease. The fact that the chemo-immunotherapy protocol can produce such regressions of overt disease at least raises the hope that it might be of value as an adjunct to surgery and prevent or delay the appearance of subclinical metastases. Such a trial is currently in progress.

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Yolk Sac Derived Teratomas and Carcinomas in Hamsters*

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Abstract—*The induction as well as the histology of visceral yolk sac derived benign teratomas in hamsters is described. The teratomas are characterized by the presence of adult well-differentiated tissues derived from all three germ layers. In these teratomas malignant transformation to yolk sac carcinoma was occasionally observed. The histogenesis of this malignant tumor is described and its origin discussed in relation to viral induced yolk sac carcinoma.*

INTRODUCTION

WE HAVE previously described the development of teratomas from displaced yolk sac in rats [1, 2]. These tumors were composed of various adult well-differentiated tissues derived from all 3 germ layers. No marked difference was recorded in the kind of tissues found at 3 weeks after the surgical procedure and those observed 6–9 weeks later. The much greater differentiation potential of the ectopic visceral yolk sac in our system compared to the limited differentiation obtained by Payne and Payne [3] who implanted the visceral yolk sac in the peritoneum may be explained by the continuity of the extra-uterine part of the visceral yolk sac with the intra-uterine one until the extension of the placenta.

The present experiments were performed in order to verify whether the differentiation of visceral yolk sac outside the uterus after fetectomy occurs in rats only, or whether it is a general phenomenon common to other species of rodents such as the hamster. We also wanted to extend the observation period until 4 months after the operation to explore the possibility of degenerative or malignant changes in the teratomas. Moreover, in order to determine if later extension of the placenta from the uterus can influence the differentiation potential of the yolk sac, we treated one group of hamsters with progesterone. This hormone is known to prolong the retention of the placenta in the uterus after fetectomy [4].

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MATERIAL AND METHODS

Hamsters of the inbred ALB/Mey Pfd strain were used. Nine days after mating, laparatomy was performed on pregnant primiparous hamsters. In both uterine horns the fetuses were removed together with the amnion and the yolk sac was gently pulled through the incision and left outside the uterus. In each place of incision two sutures were put in the uterine wall. All placentas were left *in situ* [1].

Half of the hamsters were treated with medroxyprogesterone acetate (Depoprovera, Upjohn). An injection of 25 mg was given subcutaneously on the day of fetectomy and 14 and 28 days afterwards.

Two to four months after operation the animals were killed by cardiac puncture under ether anaesthesia, and autopsy was performed.

For histological studies the uterine horns and in some cases other organs were routinely fixed in formaldehyde, embedded in paraffin and stained with erythrosine-hematoxylin and PAS. From each tumor observed 6–8 sections were prepared for histological examination.

For ultrastructural studies small pieces of any tumors were fixed in glutaraldehyde with osmium post-fixation, stained with uranyl acetate, dehydrated and embedded in araldite. Thin sections were cut on OMU-2 Reichert ultramicrotome, stained with lead hydroxide and examined with a Zeiss 9S electron microscope. A few one micron thick sections were cut from each block and stained with methyl blue and safranin.

RESULTS

Macroscopic appearance

Fifty-nine out of 60 operated hamsters developed extra-uterine tumors attached to the

uterine horns. The number of tumors varied from 1 to 5 per uterus, a total of 197 tumors being recorded among 50 operated animals. The size of the tumors varied from 0.2 to 2 cm dia. They were well encapsulated, had a smooth surface and remained attached to the uterine wall (Fig. 1). After incision many tumors displayed several cysts containing yellow mucinous fluid. No detectable lesions were observed in the other organs of these tumor-bearing hamsters.

However, in 7 cases ascites was found during autopsy. The ascitic fluid was hazy and the peritoneum was covered with small yellow to reddish tumors (Fig. 2).

Histological appearance

Histologically, the tumors were characterized by the presence of a variety of differentiated tissues (Fig. 3). Most frequently epidermal and endodermal cysts were observed. The epidermal cysts contained keratin and were lined with a well-differentiated squamous epithelium consisting of all the structural layers commonly present in mature skin (Fig. 4). In the subadjacent connective or adiposal tissue layer, skin appendages, such as hair follicles and sebaceous glands, were frequently observed.

The endodermal cysts contained mucin and were lined with a columnar epithelium which often formed folds similar to intestinal villi, with a central core of connective tissue. Smooth muscle was regularly seen around these endodermal cysts.

In many tumors cartilage and bone with bone marrow were found (Fig. 5). Striated muscular tissue was often observed. Less frequently the tumor also contained lymphoid tissues, nervous tissues, pancreas, salivary glands and thyroid (Fig. 6).

The number of different tissues present in examined tumors is summarized in Table 1. Their histological and ultrastructural appearance was identical to that previously described in the rat yolk sac derived teratomas [1].

The presence of all the described well-differentiated tissues was not dependent upon the time of tumor development (from $2\frac{1}{2}$ to 4 months), nor upon the treatment or not with progesterone.

However, during the whole observation period from $2\frac{1}{2}$ until 4 months we observed, in 24 tumors out of a total of 197, the presence of proliferating parietal yolk sac cells and their histological evolution from an *in situ* proliferation to disseminated yolk sac carcinoma.

In the histological sections made from 8 tumors which had developed for $2\frac{1}{2}$ months, small foci of epithelial cells secreting eosinophilic substance were seen inside endodermal cysts of the teratomas (Figs. 7 and 8). This eosinophilic substance was also shown to be PAS-positive and diastase-resistant.

Table 1. Presence of different tissues in yolk sac derived teratomas developing during $2\frac{1}{2}$ to 4 months

	Months after fetectomy				Total number
	$2\frac{1}{2}$	3	$3\frac{1}{2}$	4	
Number of tumours examined	36	41	63	57	197
Various tissues observed					
Epidermal cyst	31	37	56	49	173
Skin appendages	21	26	33	21	101
Endodermal cyst	22	26	48	34	130
Muscle	17	24	41	31	103
Cartilage	11	17	29	18	75
Bone	9	13	20	14	56
Bone marrow	7	11	19	10	47
Lymphoid tissue	4	5	8	5	22
Nervous tissue	4	6	3	2	15
Pancreas	1	1	3	1	6
Salivary gland	3	5	5	4	17
Thyroid	1	1	2	—	4
Parietal yolk sac proliferation	8	—	—	—	8
Yolk sac carcinoma *in situ*	—	9	—	—	9
Yolk sac carcinoma with metastases	—	—	3	4	7

In 9 tumors examined after 3 months these foci were found to be larger and frequently similar in structure to the ascitic form of yolk sac carcinoma. They were composed of endodermal cells which surrounded a hyalin substance. In some cases these foci consisted of large aggregates of polygonal cells with one, or occasionally two, pleomorphic nuclei and cytoplasm containing PAS-positive droplets. The same PAS-positive material was found between the cells. These foci were present only inside the teratomas (Fig. 9), but never in the uterine wall.

In 7 cases, killed $3\frac{1}{2}$ or 4 months after fetectomy, numerous metastases were found in the peritoneum, lymph nodes, lungs and under the liver, or kidney capsule. Also, at that later stage malignant cells were never observed in the uterus itself. The structure of these malignant neoplasms as well as their metastases was identical to the previously described yolk sac carcinoma in rats (Fig. 10) [5].

Electron microscopy

The ultrastructural studies of metastatic tumors from 2 hamsters showed the presence of endodermal cells embedded in lamellar substance-hyalin. The cytoplasm of the cells was

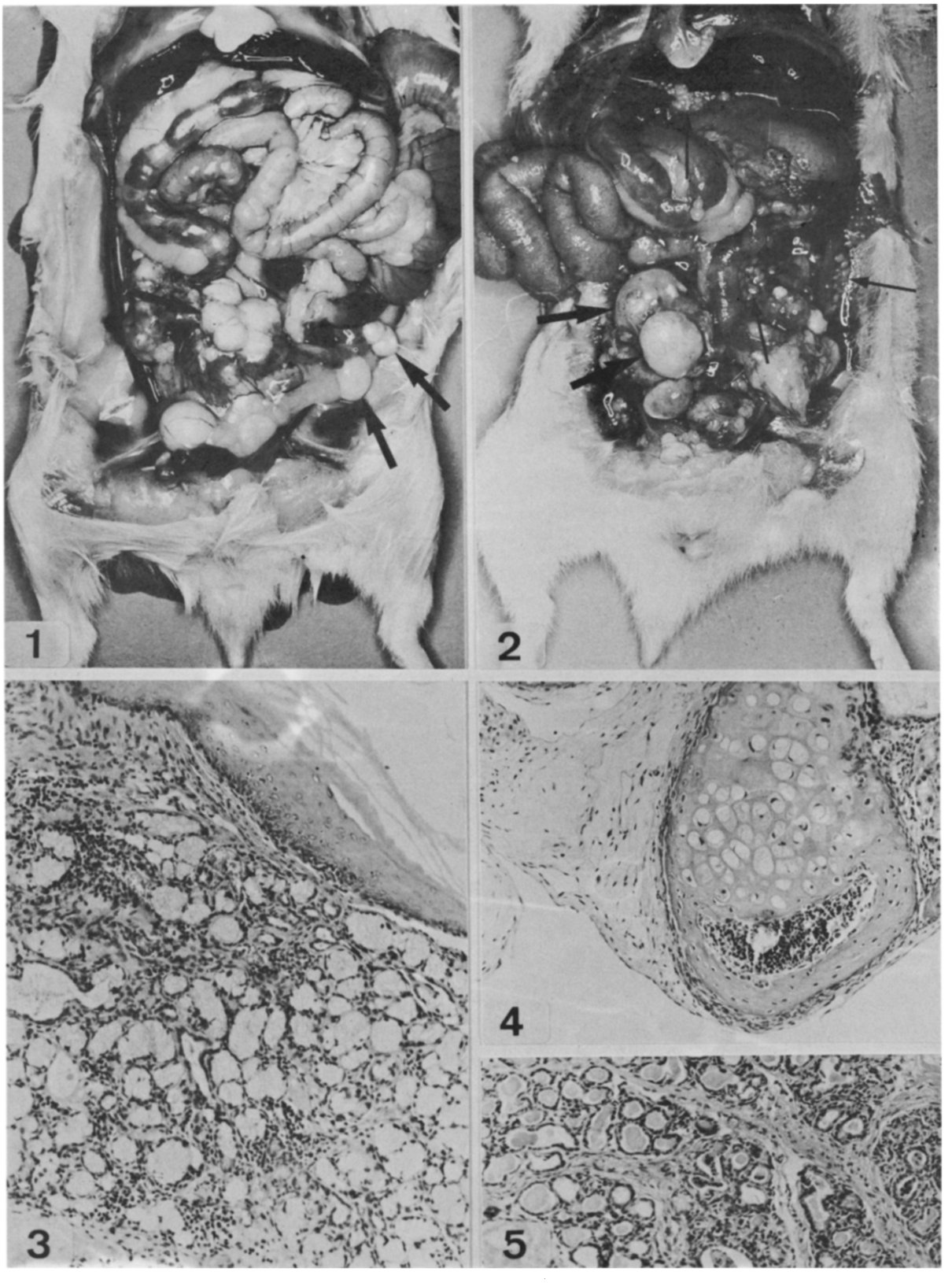

Fig. 1. Macroscopic appearance of multiple teratomas attached to the uterine horns (arrows).

Fig. 2. Macroscopic appearance of teratomas (thick arrows) and disseminated yolk sac carcinoma (thin arrows).

Fig. 3. Histological appearance of wall of cyst composed of squamous epithelium and structure of salivary glands. H and E × 110.

Fig. 4. Part of teratoma containing cartilage, bone and bone marrow. H and E × 110.

Fig. 5. Thyroid tissue in teratoma. H and E × 110.

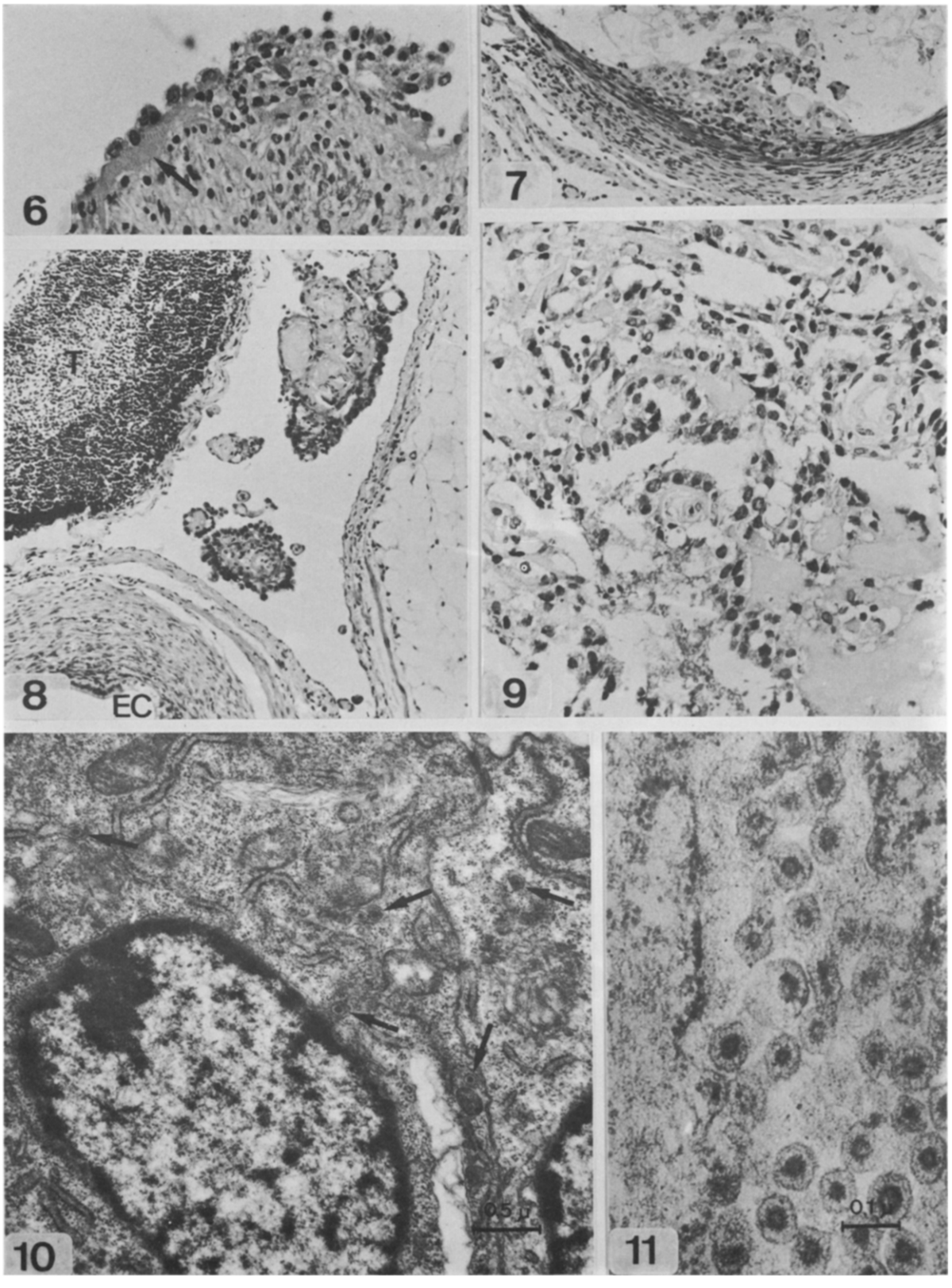

Fig. 6. Part of cyst lined with endodermal cells secreting hyalin substance (arrow). H and E × 280.

Fig. 7. Part of cyst with proliferation of parietal yolk sac cells. H and E × 110.

Fig. 8. Focus of yolk sac carcinoma cells between well-differentiated tissue of teratoma. T = thymus, EC = endodermal cyst. H and E × 110.

Fig. 9. Metastatic yolk sac carcinoma. H and E × 280.

Fig. 10. Ultrastructure of yolk sac carcinoma cell with well-developed rough-surfaced endoplasmic reticulum and numerous free ribosomes. Intracisternal virus-like particles are seen (arrows).

Fig. 11. Numerous "spoked" particles with centrally placed nucleoid attached to the bilaminar membrane by rays.

rich in rough-surfaced endoplasmic reticulum and free ribosomes were often seen (Fig. 11). Many cells contained endoplasmic cisternae filled with a grey substance. The structure of these tumors was very similar to the previously described yolk sac carcinoma in the rat.

However, in many cells we found intracytoplasmic virus-like particles. These particles were enclosed, either singly or in groups, within small vacuoles, which often had ribosomes on their membranes and were, presumably, part of the granular endoplasmic reticulum (Fig. 11). The particles had a mean outer diameter of approximately 100 mμ and were limited by a bilaminar membrane to which a centrally placed nucleoid was attached by a number of characteristic rays or spokes (Fig. 12).

Tumor transplantation and tissue culture

In 5 cases, we attempted to transplant the encapsulated tumors or metastatic tumors into young adult hamsters by subcutaneous trocar implantation. Three encapsulated tumors, which proved afterwards to be teratomas devoid of malignant parietal yolk sac cells, did not grow out after transplantation. The metastatic tumors were successfully transplanted in this way and were kept as continuous transplantable lines in syngeneic hamsters. Their microscopic appearance after transplantation was identical to that of the metastatic tumors, which were diagnosed as yolk sac carcinoma.

DISCUSSION

The results described indicate that the displaced visceral yolk sac in hamsters possesses the same capacity of differentiation as in rats and, as we have recently described, in mice [6]. This membrane, placed outside the uterus after fetectomy, gives rise to teratomas containing various well-differentiated tissues, derivatives of all 3 germ layers. Other teratomas which also contain derivatives of all 3 germ layers are the testicular teratomas, described by Stevens [7–9], derived from primordial germ cells and the ovarian teratomas derived from parthenogenetically activated ovarian eggs [10]. In these tumors, the stem cell of the teratoma is a totipotential germ cell. Such multipotential cells (primary germ cells) are formed in the visceral yolk sac during embryogenesis and migrate to the genital ridges. Thus, one has to bear in mind the possibility that some of these cells do not leave the yolk sac until the day of fetectomy and therefore may be responsible for the development of the teratomas we have described.

In our previous papers [1, 2] we gave the arguments against germ cell origin of the yolk sac derived teratomas. However, definite proof was obtained by treating pregnant rats with busulphan. This drug, which is known to destroy the germ cells during their migratory phase, led to the complete absence of these cells in the gonads [11–13]. However, busulphan did not influence the number or the morphology of the yolk sac derived teratomas. Therefore, it can be concluded that these teratomas do not arise from germ cells [14].

The presence of derivatives of all three germ layers in teratomas is observed only when the operation is performed early enough: in rats at day 12 and in mice at day 11 [6]. The same differentiation potentiality of the visceral yolk sac in hamsters is observed at day 9 of pregnancy.

As indicated by our results, the capacity of differentiation does not depend upon the time of expulsion of the placenta from the uterus, which is delayed by hormonal treatment. Indeed, no difference was found in the development or structure of teratomas in the hamsters treated or not with progesterone. This proves that 7 days connection of the yolk sac with the placenta (the period of gestation in hamsters being 16 days) is sufficient to start the differentiation which will lead to the development of various well-differentiated tissues. This is in agreement with the findings of our previous studies where we observed the first steps of differentiation as early as 4 days after fetectomy [2].

Moreover, we did not find any difference in quality or quantity of differentiated tissues in teratomas which had developed for $2\frac{1}{2}$, 3, $3\frac{1}{2}$ or 4 months, with the exception of parietal yolk sac proliferation. In the teratomas of hamsters sacrificed $2\frac{1}{2}$ and 3 months after fetectomy we observed, respectively, proliferation of parietal yolk sac and yolk sac carcinoma *in situ*. In seven out of 26 hamsters killed $3\frac{1}{2}$ or 4 months after operation we found disseminated yolk sac carcinoma. However, it was very difficult to find a primary tumor, only microscopic examination of teratomas showed the presence of malignant proliferation in endodermal cysts. Therefore, it can be concluded that in the cysts of teratomas in which endodermal proliferation occurs in some cases the proliferating cells are hyalin-secreting parietal yolk sac cells. This seems likely if one bears in mind that the endoderm of visceral yolk sac can also differentiate into various other types of epithelium such as intestinal, bronchiolar and transitional epithelium. All these epithelia although characteristic for embryo proper, differentiate in these teratomas from visceral yolk sac. As it was demonstrated that transplantation of embryo proper under the kidney capsule may

give rise to yolk sac carcinoma [15], it is probable that embryonal parts can differentiate into extra-embryonal ones and that, conversely, the fetal membranes can give rise to tissues which usually develop in the fetus. Moreover, these yolk sac derived endodermal cysts in teratomas can proliferate and form a structure similar to parietal yolk sac and after that yolk sac carcinoma.

Until now it was thought that yolk sac carcinoma in rodents differentiates from teratocarcinoma. Such differentiation was observed *in vivo* as well as *in vitro* in spontaneous or induced teratocarcinoma in mice [7, 8, 16–19] or by MSV inoculation in placentas of fetectomized rats [5, 20]. Comparing our last and present experiments one can argue that yolk sac carcinoma can develop in the uterus of fetectomized animals without virus inoculation. Such a conclusion was reached by Sakashita and Hirai [21]. It seems, however, that the origin of the yolk sac carcinoma in the case of virus inoculation into placenta after removal of the fetus as well as the free part of the visceral yolk sac which does not adhere to the placenta is different from those cases where the fetal membranes are left outside the uterus without virus inoculation.

The MSV induced yolk sac started to proliferate in the uterus itself and developed from parts of the yolk sac which adhered to the placenta. In some cases we could even observe embryonal carcinoma which differentiated into yolk sac carcinoma [22]. All these tumors metastasized 2–3 months after fetectomy.

The yolk sac carcinomas described in the present experiment start as proliferations in endodermal cysts in teratomas and the metastases appear not earlier than $3\frac{1}{2}$–4 months after operation. Moreover, we never found tumoral structures in the uterus itself which proves that these tumors develop in differentiated teratomas. The teratoma-derived yolk sac carcinomas can be compared to human malformation teratomas which may become cancerous, and MSV-induced yolk sac carcinomas to human tumoral teratomas which arise from multipotential cells [23].

In the cytoplasm of the yolk sac carcinomas described here, we found virus-like particles of unusual "spoked" morphology. Similar particles have been observed in hamster tumors and in tumor lines cultured *in vitro*, but the significance of these viruses is not known [24]. The localisation of these particles is very similar to intracisternal A-type particles which have been described in mouse teratocarcinoma and mouse yolk sac carcinoma [25, 26] as well as in mouse embryo [27]. They are thought to play a role similar to that of fetal antigen [28].

Preliminary results indicate that the intracisternal "spoked" virus is present not only in tumors but also in hamster embryos but is absent in the normal tissues of the adult hamster. The role of these particles seems worth further investigation.

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Pulmotropic Carcinogenic Activity of *N*-Methyl-*N*-Nitrosopropionamide*

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Abstract—*N-Methyl-N-nitrosopropionamide applied intravenously (i.v.) to BD-rats in small weekly repeated doses selectively induced lung cancer in a high yield. Moreover, like the well-known pulmonary carcinogen N-methyl-N-nitrosourethane, N-methyl-N-nitrosopropionamide avidly reacts with cysteine, whereby elementary nitrogen is evolved. On the other hand, N-methyl-N-nitrosourea, a powerful neurotropic carcinogen, does neither react with cysteine nor induce lung cancer. It has been, therefore, concluded that reaction with cysteine under concomitant N_2-liberation might be a characteristic feature of lung cancer-inducing nitrosamides; accordingly all nitrosamides which display this reaction should on i.v. application produce lung cancer and vice versa. To check this hypothesis, N-methyl-N'-nitro-N-nitrosoguanidine was chosen. A report on these experiments, which yielded a high percentage of malignant lung tumours, will be given later.*

INTRODUCTION

IN THE large class of *N*-nitroso compounds the carcinogenic activity of which was extensively investigated in rats by Druckrey and his school [1–3] beside numerous nitrosated derivatives of carbonic acid (nitrosoureas and nitroso carbamic esters) some nitrosocarboxylamides were also studied [1, 2]. Upon oral administration they induced cancer of the forestomach [1, 2]. The same effect was achieved with some orally applied nitrosoureas and nitroso carbamic esters, e.g. *N*-methyl-*N*-nitrosourethane (MNUT) and *N*-methyl-*N*-nitrosourea (MNU). However, in spite of having this property in common, MNUT and MNU given intravenously in small weekly doses strikingly differ in their specific organotropic properties: they induce selectively lung cancer and brain tumours respectively [1]. It interested, therefore, whether or not organotropic effects and of what kind would be found after i.v. application of nitrosocarboxylamides. *N*-Methyl-*N*-nitrosopropionamide (MNPA) was chosen for this purpose.

The reactivity of *N*-nitroso compounds and their derivatives with cysteine has interested us for some years [2, 4]. As the first preliminary experiments have shown a very rapid gas liberation and decomposition of MNPA upon addition of cysteine, it was decided to investigate this reaction in some detail.

MATERIAL AND METHODS

N-Methyl-N-nitrosopropionamide

MNPA was first synthesized by R. Preussmann by nitrosation of *N*-methylpropionamide [5]; we have later modified the synthesis in a few details.

The readily water-soluble methylpropionamide (prepared by adding propionyl chloride dropwise to a cooled alkaline solution of methylamine hydrochloride) was isolated from the reaction mixture by adding excess of solid potassium carbonate; it then appeared as a white layer and could be separated, dried and distilled. Nitrosation was performed by slowly adding of H_2SO_4 to a cooled aqueous solution of the amide and nitrite.

By using fluorescent thin layer plate (TLC Aluminium sheets, Silica gel 60 F 254 precoated, layer thickness 0.25 mm; E. Merck, Darmstadt; as solvent benzene was used) and exposing the chromatogram briefly to N_2O_3 and its dissociation products [6] it was possible to show in u.v.-light that MNPA thus obtained was still contaminated with un-nitrosated methylpropionamide (besides containing other impurities). On a silica gel column (Silica gel 60, 70–230 mesh ASTM, E. Merck, Darmstadt; ben-

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‡Dissolved in 1973: the reported experiments were performed in 1970–1973.

zene was used as eluent), however, the substance could be purified; after vacuum distillation only a single spot appeared on the thin layer chromatogram.

Elementary analysis was in accordance with the formula assumed for the substance. (Found: C, 42.00; H, 6.90; N, 24.40%. Calc. for $C_4H_8N_2O_2$: C, 41.37; H, 6.94; N, 24.13%).

MNPA is a yellow–red fluid (b.p. 43.5°C/11 mm Hg). It is soluble in organic solvents but only moderately in water (1.1%). In the visible region the absorption spectrum in methanol has the maximum at 402 nm ($\varepsilon_{max} = 114$); for the u.v. range, λ_{max} at 238 nm ($\varepsilon_{max} = 9300$) was observed.

The volume of the gas liberated in the course of reaction between MNPA and cysteine at 27°C was measured in the Warburg apparatus. In the main compartment of the Warburg flask 2.8 ml of a 0.04 M cysteine solution in M/15 phosphate buffer (KH_2PO_4–Na_2HPO_4) were added (pH = 6.0); 0.2 ml of MNPA solution (0.02 M) in the same buffer were put into the side arm of the vessel which thus contained 4 μmole of MNPA. To prevent the autoxydation of cysteine the air had to be displaced by flushing the flask for 10 min with a stream of N_2 from a steel cylinder. After temperature equilibration, the content of the side arm was tipped into the main compartment and manometer readings recorded at regular time intervals, taking into account thermobarometer corrections. To detect any possible evolution of CO_2, the reaction between cysteine and MNPA was allowed in some experiments to proceed in the presence of 10% KOH, put into the center well of the flask together with strips of filter paper. Simultaneously, the decomposition of MNPA in absence of cysteine was registered.

In experiments aimed at testing the inhibition of the reaction between cysteine and MNPA by inhibitors of SH-groups, the solution in the main compartment besides cysteine (0.04 M) contained also iodoacetamide (0.1 M) and N-ethylmaleimide (0.08 M) respectively.

Experimental animals

For animal experiments female and male BD X-rats [7] were used. Age: 3 months, average body weight 220 and 350 g for females and males respectively. Standard food was alternating Altromin maintenance diet 1320 and Latz biscuits; water ad libitum. Characteristic for BD-rats is a low spontaneous tumour rate; at the age of 2 yr the incidence of benign and malignant tumours is about 2 and 2% respectively [7]. Spontaneous lung tumours are hardly ever observed.

Determination of LD_{50} was performed on several groups of 6 rats; MNPA was dissolved in normal saline and injected intravenously (0.5%). As the BD-rats are very homogeneous, the lethality regression lines display a high slope; the LD_{50} could be, therefore, determined graphically.

To test the carcinogenic activity of MNPA, 24 rats of both sexes were given i.v. 2 mg/kg weekly, as 0.2% solution in normal saline. After 31 weeks the treatment was discontinued. The total dose given thus amounted to 62 mg/kg body weight.

The experimental animals were subjected to complete autopsy immediately after natural death; some were sacrificed in the final stage with chloroform. Removed organs were fixed in 10% formalin. Organ slices were stained with hematoxylin and eosin for microscopic examination.

For quantitative evaluation of the carcinogenesis experiment the method of Miescher *et al.* [8] for the calculation of the cumulative percentage mortality was used.

RESULTS

Toxicity

The value of LD_{50} determined for MNPA by the i.v. route was 15 mg/kg. At the autopsy of the animals, which died after administration of the median lethal dose, severe haemorrhagic pulmonary oedema was regularly observed; pathological alterations of other organs were only slightly indicated or absent. The median survival time was 7 days.

Carcinogenesis experiments

In animals treated intravenously with 2 mg/kg MNPA (13.3% of LD_{50}) per week, lung tumours were found in a high yield (84%). Tumours of other organs—especially of the brain—were not observed.

Before the occurrence of the first tumour 5 rats died of pneumonia. Of the surviving 19 animals 13 developed lung cancer; 6 rats died intercurrently of lung infections. Using logarithmic probability paper a linear regression with high slope was obtained after plotting cumulative percentage mortality against individual survival times of rats with tumours (Fig. 1). Evidently, the individual survival times are lognormally distributed. The mean survival time was 420 days, standard deviation ± 100 days. Since lung tumours spontaneously hardly ever occur in BD-rats, these findings evidence a specific pulmotropic carcinogenic action of MNPA.

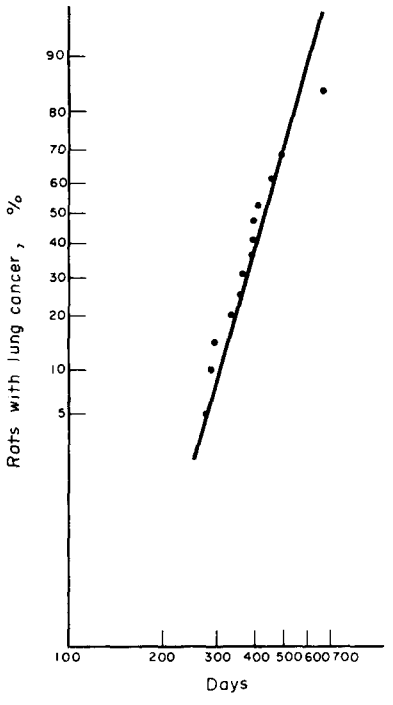

Fig. 1. Survival times of rats with lung cancer. Each point represents an animal, which died of a tumour. Ordinate: cumulative percentage mortality. Abscissa: individual survival time, days.

Either multilocular tumour growth or solitary nodes were found at the autopsy (Fig. 2); metastases of the mediastinal lymph nodes were frequently observed. On microscopic examination all tumours proved to be carcinomas, but of different histological types. Squamous cell carcinomas (with and without cornification) were encountered in 7 cases; in 5 cases alveolar-cell carcinomas were found. Finally, an anaplastic carcinoma was registered. Pronounced pleomorphism and anaplasia were always detectable at least in some parts of the tumours.

Decomposition of MNPA in the presence of cysteine

By using specific absorption reagents (10% NaOH; 10% $Na_2S_2O_4$ in 10% NaOH; CuCl in ammoniacal NH_4Cl-solution i.e. Winkler's reagent) the gas evolved in the reaction between MNPA and cysteine was shown not to be either CO_2, O_2 or CO; since it was insoluble in ethyl ether, it could not have been diazomethane, CH_2N_2. Furthermore, no precipitate of cystine was formed during the reaction; the possibility of N_2O formation seems thus to be excluded [9]. Considering the structural formula of MNPA, the conclusion that N_2 is liberated seems to be justified.

In the Warburg apparatus the volume of N_2 evolved could be exactly measured. At pH 6.0 solution of MNPA in phosphate buffer slowly decomposes under gas liberation. Upon addition of cysteine, however, a very rapid gas evolution takes place (Fig. 3). The volume of N_2 given off amounted to 85% of that which would be expected if the whole N of MNPA molecule had been liberated as N_2 (i.e. 89.6 μl N_2 at STP, corresponding to 4 μmole MNPA).

No concomitant evolution of CO_2 could be detected. A clear dependence of decomposition rate on pH was observed; both in the presence and in the absence of cysteine liberation of N_2 proceeded at pH 7.2 substantially faster than at pH 6.0. Reduction of cysteine concentration to 0.01 M retards the disintegration of MNPA. Decomposition products have not been identified yet.

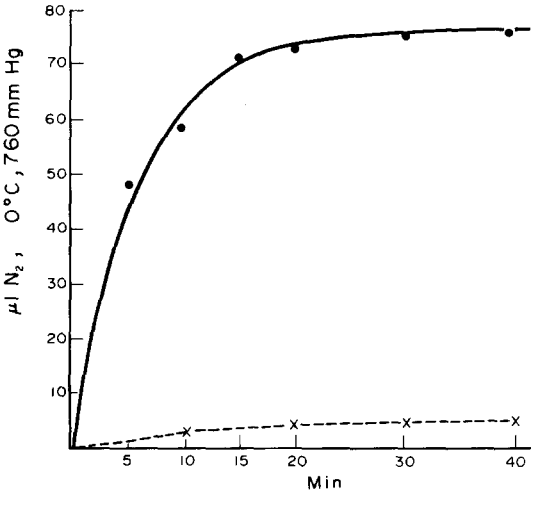

Fig. 3. Time course of evolution of N_2 from MNPA in the presence or absence of cysteine. The solid line was calculated by the method of least-squares.
Points: manometer readings.
The reaction mixtures contained 4 μmole MNPA and 112 μmole cysteine in $M/_{15}$ phosphate buffer ($KH_2PO_4 - Na_2HPO_4$), pH 6.0, at 27°C.
Broken line: decomposition of MNPA (4 μmole) in the same phosphate buffer without cysteine; pH 6.0, 27°C.

By the method of integration [10] it could be shown that the gas evolution of MNPA in the presence of cysteine represents a first-order reaction. It seems that because of the 28-fold molar excess of cysteine over MNPA the reaction is pseudounimolecular and, therefore, obeys the first-order kinetics. Calculated by the method of least squares the best value of the rate constant was found to be $k=0.17$ min^{-1}. By using inhibitors of SH-groups it was possible to show that the thiol group of cysteine was essential for the reaction with MNPA. Iodoacetamide (0.1 M) or *N*-ethyl-maleimide (0.08 M) was able to suppress the evolution of N_2 entirely. Obviously the free thiol groups are indispensable for liberation of N_2.

DISCUSSION

The results presented clearly point out two characteristic properties of MNPA: its specific pulmotropic carcinogenic action on i.v. application and its high reactivity with cysteine. In connection with these facts it seems to be important that MNUT beside inducing lung cancer also avidly reacts with cysteine with liberation of N_2 [11]. MNU, on the other hand, which given intravenously selectively induced brain tumours but never lung cancer, does in neutral aqueous media not react with cysteine [2, 12]. Considering these 3 substances, a parallelism between the pulmotropic cancerogenic action and reactivity with cysteine seems to exist. This parallelism may be purely accidental or it may be indicative of a causal relationship between the two phenomena. The latter possibility seems to be more probable. Schoental [13] has shown that *in vitro* cysteine substantially enhances the alkylation of DNA by MNUT. Like cysteine *in vitro*, tissue SH-groups in the lung may enhance alkylation of DNA by MNUT and MNPA respectively, thus triggering the process of carcinogenesis. It is, therefore, tentatively assumed that the reaction with SH-groups *in vivo* represents in the system: lung tissue—nitrosamide an essential prerequisite for induction of tumours. The failure of MNU to induce lung cancer would thus be due to its inability to react with SH-groups.

For haemodynamical reasons, the intravenously injected MNPA may be largely retained and decomposed by the lung tissue and probably only a fraction of the applied dose should reach other organs. This unequal distribution of MNPA would at least partly account for its selective pulmotropic carcinogenic activity upon i.v. application.

Moreover, there is ample experimental evidence that alkylation of DNA does not necessarily mean cancerisation [14]. For the malignant transformation of a cell by an alkylating agent much more than a merely random alkylation of the genetic material seems to be required. We have, therefore, to assume that some relevant segments of DNA—"Duplikanten" according to Druckrey [15]—must be alkylated if a normal cell is to become a cancer cell. In the lungs presumably the spatial arrangement of SH-groups (e.g. that of the non-histone nuclear protein) might be such as to enable the carbonium ions, originating from the reaction between MNPA and sulfhydryl groups, to approach and attack the relevant segments of the double helix. In the organs other than lungs a different situation may be present and even if some reaction between MNPA and SH-groups should take place, the malignant transformation cannot occur.

The fact that no lung cancer can be induced following chronical oral administration of either MNPUT or MNPA seems to indicate that under the conditions of the chronical experiment (carcinogen administered in the drinking water) both substances might be largely destroyed in the intestine, before entering the circulation; thus lung cancer cannot be induced. This presumption is supported by the fact that the LD_{50} determined by the oral route is substantially higher than LD_{50} i.v. [1], which holds true for both substances. MNU, on the other hand, being well absorbed when given orally—as evidenced by the fact that LD_{50} oral equals to LD_{50} i.v. [1]—induces in rats brain tumours also upon administration in drinking water [1].

Upon chronical oral application, however, all 3 nitrosamides induced cancer of the forestomach; thus the specific organotropic carcinogenic activity, so well pronounced upon i.v. administration of these substances, gets lost when they are allowed to act directly upon the epithelial cells of the forestomach. Whether or not the biochemical mechanism underlying the induction of the cancer of the forestomach resembles that involved in the carcinogenesis following the i.v. application of the respective nitrosamide, must at present remain unclear.

If the hypothesis outlined above should hold true, the reaction *in vitro* with cysteine under concomitant liberation of N_2 would be characteristic for all nitrosamides which would induce lung cancer following i.v. administration, thus offering a method of finding new pulmonary carcinogens in the group of nitrosamides by means of a test *in vitro*. On the other hand, the hypothesis can be checked by animal experiments. According to a suggestion of H. Druckrey and G. Eisenbrandt, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was chosen for this purpose. This nitrosamide is known to react avidly with cysteine with liberation of N_2 [9, 16]. According to the above hypothesis it should on i.v. application induce lung cancer.

A report on experiments with MNNG (performed together with J. Gimmy) which in fact yielded a high percentage of lung cancer (about 70%), will be given later.

Acknowledgements—I thank Prof. Druckrey for numerous fruitful discussions. The expert technical assistance of Mrs. Sigrid Mücke and Mr. W. Mössner is gratefully acknowledged.

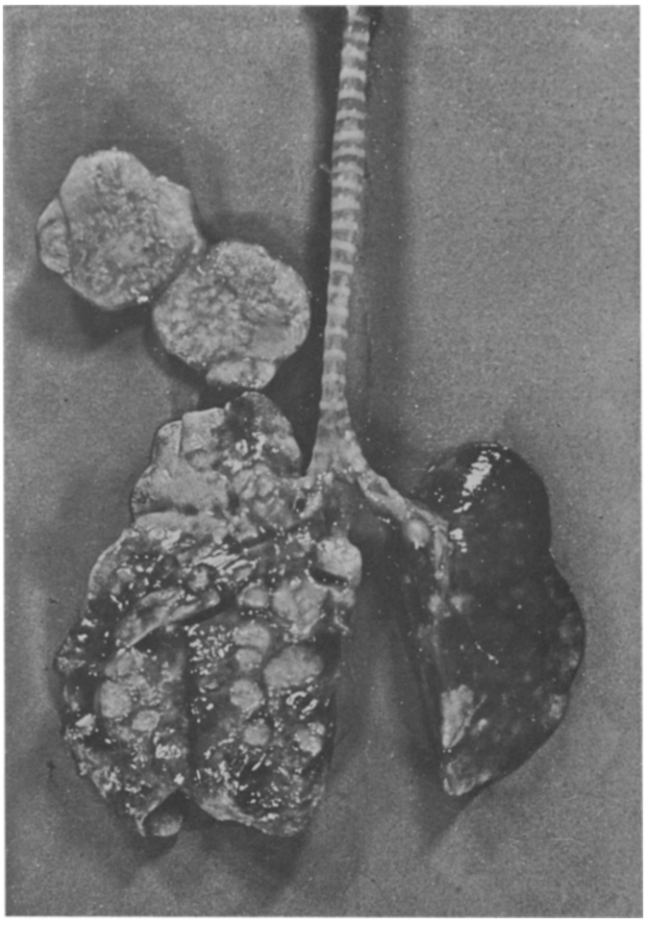

Fig. 2. Lung cancer, induced by MNPA (2 mg/kg *i.v. per week*). *Total dose:* 62 mg/kg. *Death* 290 *days after beginning of treatment. Histologically, the tumour was a squamous cell carcinoma. Next to the trachea: massive metastasis of the cancer in a mediastinal lymph node.*

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Letter to the Editor

The Effect of Temperature During the Diethylnitrosamine Treatment on Liver Tumorigenesis in the Fish, *Oryzias latipes*

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AFTER the first observation on hepatic carcinoma in hatchery-reared rainbow trout in the United States [1], experimental liver carcinogenesis in salmonid fish with several carcinogens have been studied extensively [2, 3]. However, the experiment with these fish require much space and special facilities, so small aquarium fish have been used for experimental carcinogenesis. Stanton first succeeded in the induction of liver neoplasia in the small aquarium fish, *Brachydanio rerio* [4], and after that, liver tumors in guppy have been reported [5, 6]. Recently hepatomas induced by diethylnitrosamine in the medaka (*Oryzias latipes*) has been reported by Ishikawa *et al.* [7].

Since body temperature can be controlled in fish, a series of experiments to ascertain the temperature effects on tumorigenesis at various stages has been carried out. The results of the first experiment will be reported here.

One hundred and eighty fish of both sexes of medaka (orange–red variety), about 1 yr old, 2.0–3.0 cm in body length and fed on Tetramin (Tetra Werke, West Germany), were divided into 2 groups, and each group was divided again into 3 subgroups. The first group (G1) was kept at room temperature (25 ± 2°C), and the other group (G2), at a low temperature (8 ± 2°C), during the treatment period of diethylnitrosamine (DENA, Tokyo Kasei Co., Ltd., Tokyo). DENA was added to the water in both groups at the concentrations of 100, 50 and 0 ppm for 8 weeks. After the DENA treatment at high or low temperatures, all fish were transferred to normal water and kept at 25 ± 2°C. The glass containers were set under the fluorescent light. The water was changed once a week and not aired throughout the experiment. Twelve weeks after the beginning of the experiment, the surviving fish (in G1, 19/30, 22/30 and 20/30 at the concentrations of 100, 50 and 0 ppm of DENA, and in G2, 10/30, 11/30 and 16/30 respectively) were killed and fixed in Bouin's solution. After the samples were transferred into 70% alcohol, the ratios of liver weight to body weight were examined (Fig. 1). Histological serial sections of the liver were made by a routine procedure and stained with hematoxylin and eosin.

A marked enlargement of the liver was observed macroscopically in all fish (13 fish were observed) in the high-temperature group (G1) treated with 100 ppm of DENA. The ratios of

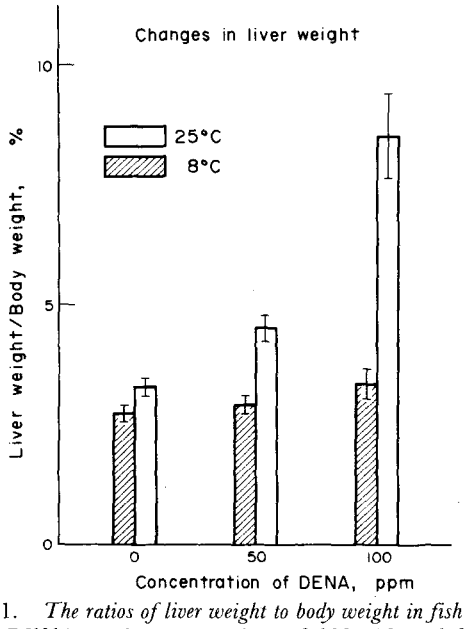

Fig. 1. *The ratios of liver weight to body weight in fish treated with DENA at the concentrations of* 100, 50 *and* 0 ppm *respectively. The ratio of fish treated at* 25 ± 2°C (*G*1) *was significantly higher* ($P < 0.001$) *than that of fish treated at* 8 ± 2°C (*G*2). *After the DENA treatment, fish of both groups were kept at* 25 ± 2°C *for* 4 weeks.

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liver weight to body weight in G1 was significantly higher ($P<0.001$) than that in G2. Many large or small tumor nodules were found through microscopic observation in all these fish (Figs. 2, 3). These tumor nodules consisted of hyperbasophilic cells with round to oval nuclei of various sizes, all containing a single prominent nucleolus. The cell density was very high in these nodules. Mitotic figures were noted frequently (Figs. 4 and 5). Some nodules appeared to be "trabecular hepatoma" and "liver cell carcinoma", and some were poorly differentiated nodules. Although the half of fish treated with 50 ppm of DENA in G1 did not have such hyperbasophilic nodules (12 fish were observed), they showed the livers associated with hyperplastic foci and the disarrangement of parenchymal cells. The infiltrating cells which were similar to lymphatic cells and/or the cells of connective tissue were also observed.

In the low-temperature group (G2), however, the majority of fish had no nodules even if 100 ppm of DENA was administered. A widely spread necroses and the infiltration of the cells mentioned above were observed frequently. However, the residual part of these livers seemed to be normal (Fig. 6). Only one fish treated with 100 ppm of DENA at low temperature had a very small hyperbasophilic cell mass (diameter $=70\ \mu m$) which was very different in size from the nodules in G1 (Fig. 7).

From these results, it is considered that the histological changes observed in fish treated with DENA at a low temperature are induced by the toxicity of the drug, and that the step to tumorigenesis is a different event from this phenomenon. In liver of fish, nitrosamine may be metabolized to some degree as Montesano *et al.* reported [8], and the various damages produced by DENA, perhaps induced by the alkylation of macromolecules as the case of mammals [9], might be relevant to cytotoxic or lethal effects on cells at low temperatures, and the step to tumorigenesis seems to require an appropriate high temperatures. In order to ascertain the cause of the difference in tumor formation between the two temperature groups, further studies are in progress.

Acknowledgements—We would like to thank Dr. T. Ishikawa and Dr. S. Takayama, Cancer Institute, Tokyo, for their kind instruction in histological observations. The study was supported in part by a Grant from the Ministry of Education, Science and Culture in Japan.

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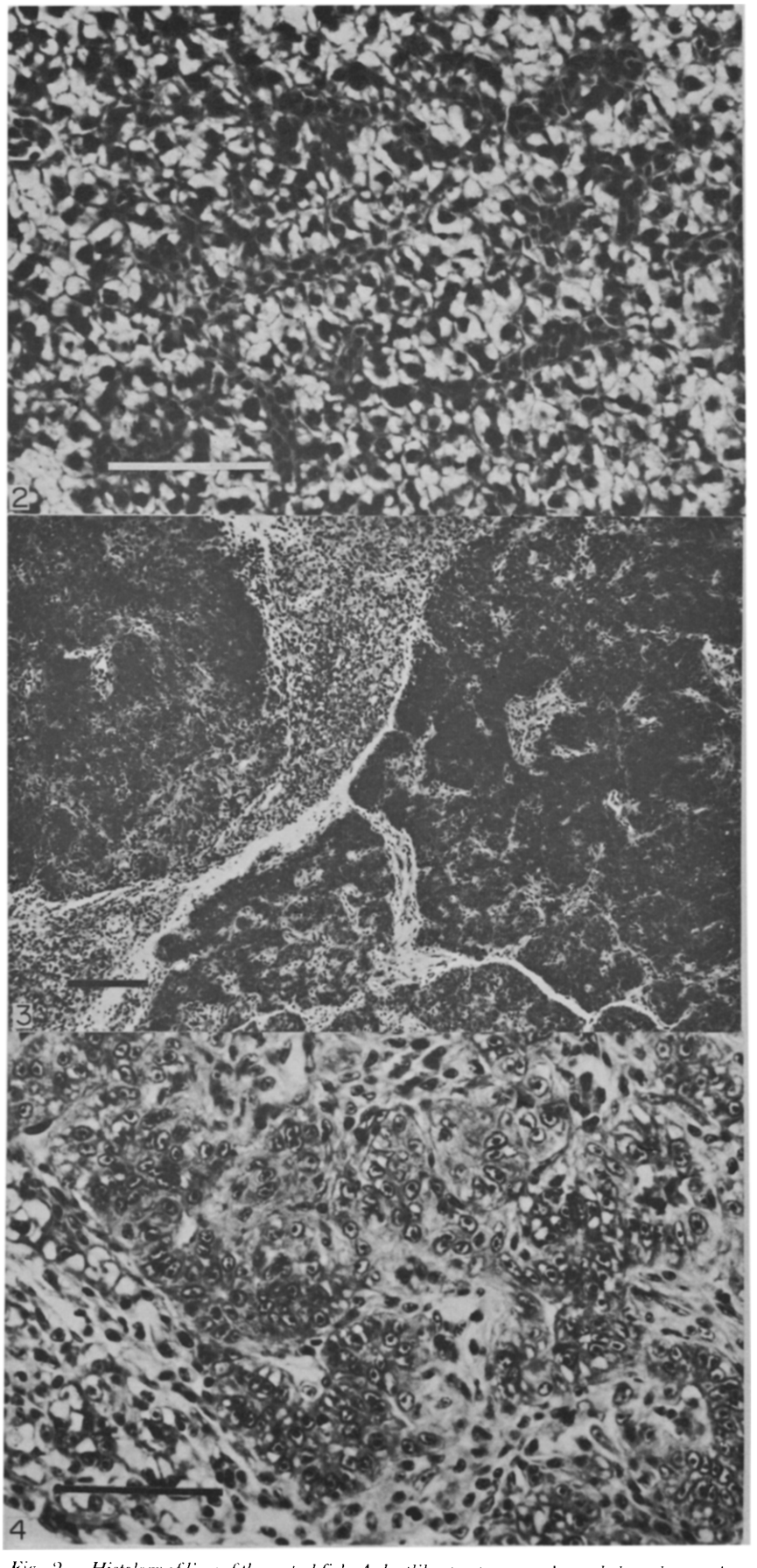

Fig. 2. Histology of liver of the control fish. A sheetlike structure was observed along the vascular sinusoids. Hematoxylin and eosin (H & E). Bottom bar, 50 μm.

Fig. 3. Liver of fish treated with 100 ppm of DENA at 25 ± 2°C for 8 weeks and then kept in tap water for 4 weeks. Large tumor nodules were observed. H & E. Bottom bar, 100 μm.

Fig. 4. High magnification of tumor nodule of the fish shown in Fig. 3. H & E. Bottom bar, 50 μm

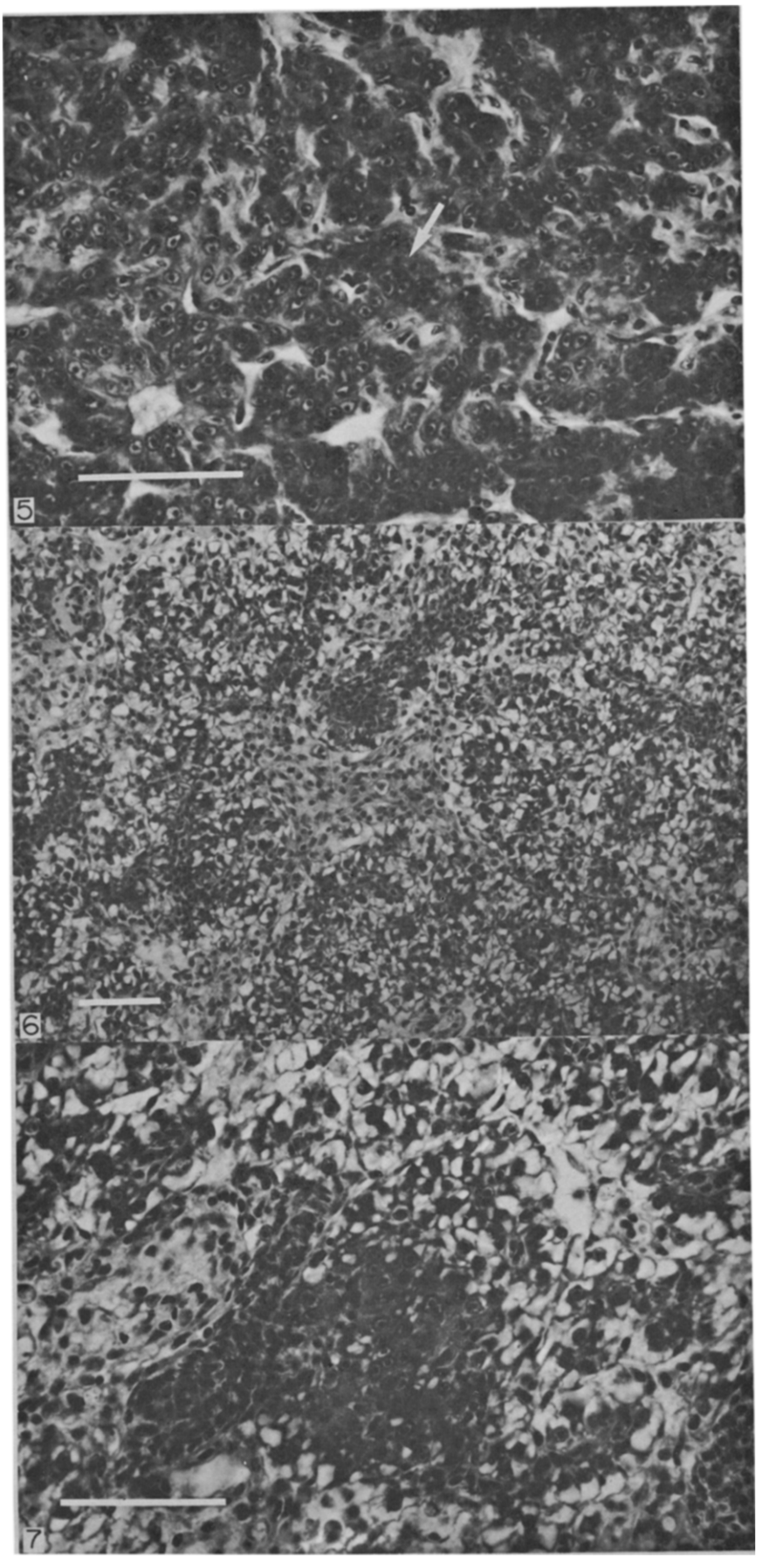

Fig. 5. Histology of typical trabecular hepatoma. This fish was of the same group as the fish shown in Fig. 3. Mitotic figure was observed (arrow). H & E. Bottom bar, 50 μm.

Fig. 6. Histology of liver of fish treated with 100 ppm *of DENA at* 8 ± 2 °C *for* 8 weeks *and then kept in tap water for* 4 *weeks at* 25 ± 2 °C. *No tumor nodule was observed. H & E. Bottom bar,* 50 μm.

Fig. 7. One experimental case of G2. The fish was treated with 100 ppm *of DENA at a low temperature and then transferred to tap water at* 25 ± 2 °C. *A small hyperbasophilic focus (dia* = 70 μm) *was found. H & E. Bottom bar,* 50 μm.

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Letter to the Editor

Inhibition of DNA Synthesis by a Factor from Ascites Tumor Cells*

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OUR PREVIOUS studies [1–3] have demonstrated that Ehrlich ascites tumor (EAT) cells release an inhibition factor for protein and DNA synthesis during incubation in an isotonic but non nutritive environment (Hank's balanced salt solution, HBSS). This synthesis inhibition factor (SIF) may be involved in the autoregulation of ascites tumor growth. Furthermore, SIF could also be directed against immunocompetent cells. Factors released by tumor cells which may allow tumor cells to by-pass the host immunological system have been described recently by other authors [4, 5]. In order to understand the possible biological significance of SIF we have investigated its effect on stimulated lymphocytes.

SIF was purified from cell-free HBSS which had been used for incubation of EAT cells. This was freeze-dried, extracted with ethanol–acetone 1:1 (v/v), and chromatographed on Biogel P2 in water. The inhibitory activity which eluted in front of the conductivity peak was used. Further purification of SIF can be achieved by paper electrophoresis in an acidic system (acetic acid–formic acid–water, 4:1:36). The gel chromatography and ultrafiltration experiments on Amicon UM2 and UM05 membranes have shown that SIF has a mol. wt in the range of 500–1000. As an assay for SIF activity we measured the depression of incorporation of radioactively labelled amino acids and thymidine into cold acid insoluble material by cells. Human lymphocytes were prepared as described elsewhere [6], cultured in medium 199 (Fa. Gibco) supplemented with 20% calf serum at a density of 2×10^6 cells/ml and stimulated by pokeweed mitogen. Seventy-two hours after addition of the mitogen, the cells were sedimented, resuspended in fresh medium and incubated for 30 min with 20 μCi/ml ^{3}H-thymidine (20 Ci/mmole). SIF was added in HBSS, the controls were supplemented by the same volume of HBSS. Incorporation of radioactivity into acid insoluble material was measured in aliquots taken at times indicated in Fig. 1. DNA synthesis in stimulated human lymphocytes is strongly inhibited by SIF. This inhibition persists throughout the observation period of 240 min. When the same experiment was carried out with EAT

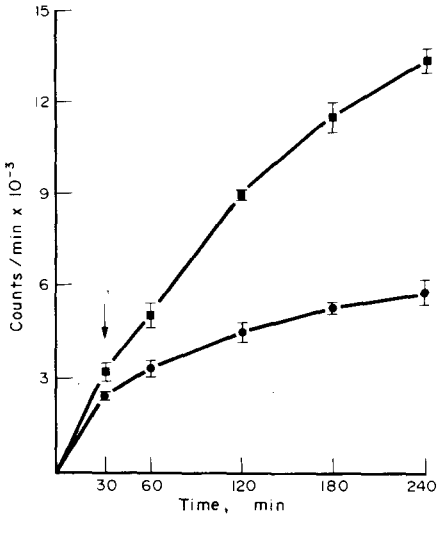

Fig. 1. Incorporation of ^{3}H-thymidine into the cold acid insoluble material of stimulated human lymphocytes. Incubates contained, per ml medium: 2×10^6 cells, 20 μCi ^{3}H-thymidine (20 Ci/mmole). Mean values and standard deviations of 3 aliquots taken at the times indicated are given. The arrow indicates time of addition of SIF. (●——●) SIF treated cells, (■——■) controls.

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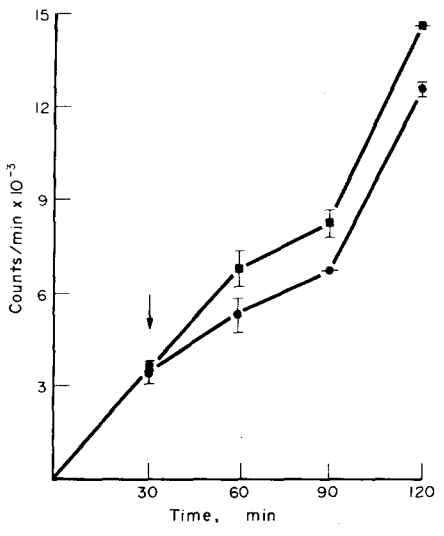

Fig. 2. Incorporation of ^{3}H-thymidine into the cold acid insoluble material of cultured EAT cells. Incubates contained, per ml medium: 5.5×10^5 cells, 2 μCi ^{3}H-thymidine (20 Ci/mmole). Mean values and standard deviations of 3 aliquots taken at the times indicated are given. The arrow indicates time of addition of SIF. (●——●) SIF treated cells, (■——■) controls.

cells which were cultured as described elsewhere [7] (Fig. 2), DNA synthesis is less affected. The same level of SIF causes an inhibition period which lasts only 30 min, after which the rate of incorporation of ^{3}H-thymidine into the treated cells recovers and parallels the control incorporation rate.

Thus, it appears that DNA synthesis is more sensitive to SIF inhibition in stimulated lymphocytes than in EAT cells. We would suggest that during the early stages of tumor development, when the number of tumor cells and, therefore, the release of SIF is low, SIF may affect primarily the immune competent cells. This is in agreement with data presented by Fauve *et al.* [4] who described factors released by tumor cells which allow tumor cells to by-pass the surveillance of the host immunological system. In a later stage of tumor development with high numbers of tumor cells, the consequently higher concentrations of SIF may act on the tumor cells themselves causing the autoregulation typical for the growth of ascites tumors [8].

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Letter to the Editor

Presence of Herpes Simplex Virus-Specific Antigens in Exfoliated Cervical Cells of Virgins and Sexually Active Women*

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SINCE the evidence for the association between cervical anaplasia and Herpes simplex virus (HSV) type 2 had been published [1], a number of investigations was devoted to clarify the role of HSV-2 in the development of cervical cancer. However, the way how the virus might be linked with the tumour remained unanswered [2].

By the indirect immunofluorescence technique the Herpes virus-specific antigens in the cervical cells can be localized [3]. As exfoliated cells are easily accessible, this method provides a feasible approach to investigate the relationship between HSV-2 and cervical cancer.

Our previous work on the presence of HSV antigen in exfoliated cervical cells revealed: (a) not only anaplastic cells, but also apparently normal cells containing the antigen [4], (b) gradual increase of the prevalence of antigens from normal to cancerous stage [5]. These studies concerned sexually active women. The question therefore emerged whether cervical cells of virgins contain the antigens or not. In this report we present our recent data on the prevalence of HSV antigens in cervical cells in the following groups of women.

Virgins. They were selected from those girls seen and examined at the Outpatient Department of Children Gynecology, Health Centre, Pécs. Their age was between 10 and 18 yr. Only the girls were involved in the study (106 in number) whose virginity was approved unequivocally by physical examinations. Patients visited the Department because of dysfunctional bleeding, vaginal fluor, uncertain origin of abdominal pain and hormonal disorders. Imprints were taken between periods.

Sexually active women. This group includes: (a) 710 women who were selected by the on-going screening programme as part of our Herpes virus–cervical cancer project. They had normal cervical epithelium by pelvic examination, Papanicolaou test and colposcopy; (b) 205 women with bland disorders. This term covers ectopium, iodine-negative areas, erosions, inflammations seen by colposcopy and histological diagnosis like keratitis, hyperplasia, metaplasia, basal activity according to definitions described previously [6], (c) sixty-two patients with cervical dysplasia diagnosed histologically and (d) 49 patients with invasive cervical carcinoma confirmed histologically.

Cervical imprints were obtained by a piece of sponge impressed to the surface of the cervical portion, atypic lesion or tumour. From each person six imprints were made on coded slides. They were fixed in cold acetone for 5 min and kept at −20°C until processed.

The presence of HSV-specific antigens in cervical cells was determined by the indirect immunofluorescence method. Imprints were treated with rabbit antiserum to HSV-2 and then with fluorescein-conjugated sheep anti-rabbit IgG from the Human Sera and Vaccine

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Table 1. Incidence of HSV antigens in exfoliated cervical cells of virgins and sexually active women

Group	No. tested	No. of positives	(%)
1. Virgins	106	None	—
2. Women with normal cervical epithelium	710	64	9.1
3. Women with bland disorders	205	79	38.0
4. Patients with cervical dysplasia	62	35	56.4
5. Patients with invasive cervical carcinoma	49	45	91.8

Institute, Budapest, Hungary. The preparation of anti-HSV-2 rabbit immune serum, and the particulars of conjugated IgG were described earlier [7]. Slides mounted with 90% glycerol were examined in a Zeiss fluorescence microscope. The test was specific for HSV antigens, as antiserum was prepared by immunizing rabbits with HSV-2 propagated in primary chick embryonic fibroblast cultures; serum used in the test was absorbed with a cell culture derived from human carcinoma of the larynx (HEp-2), HEF and HeLa cells. The serum did not show fluorescence when checked with non-infected HEP-2, HeLa, and cytomegalovirus-infected (strain AD-169) HEF cells. Absorption of the immune serum with HSV-2-infected HEp-2 cells removed almost entirely its staining capacity to HSV antigen, leaving a faint fluorescence not higher than in dilution of 1 in 5. For staining imprints a working dilution of 1 in 50 was applied. For detecting HSV-specific antigens in the cervical cells, the following staining pattern was used. Two impressions of cervical cells were treated with HSV-2 immune serum, 2 others with normal rabbit serum, and the remaining 2 with 0.1 M phosphate-buffered saline (pH 7.2). Samples were considered positive for HSV antigens when cervical cells had shown characteristic fluorescence with HSV-2 immune serum only. Evaluation was done by a technologist not aware of the code.

By this procedure we could detect antigens in none of the samples from the 106 virgins examined so far. In contrast to this, in exfoliated cervical cells of sexually active women with apparently normal, healthy cervical epithelium, HSV-specific antigens were present in 9%. Comparison of the prevalence of antigens by groups with different cervical disorders reveals that antigen positivity increases from normal to cancerous stage (Table 1). A follow-up study of selected groups of women indicated that the HSV antigen, when once acquired, remains in the cervical cells for at least 1 yr [5]. These facts may support the initiating role of Herpes virus genitalis in the development of anaplastic changes. The fact that none of the samples from virgins contained the antigens does strengthen further the hypothesis that Herpes virus infection precedes neoplastic changes of the cervical epithelium. Moreover it underlines that sexual activity is indispensable for acquiring HSV antigens.

Since non-cytopathogenic, cell-associated virus is capable of transforming cells *in vitro* [8], we suppose that healthy women with herpes virus antigens in their cervical cells stand a certain risk of developing cervical cancer. We suggest that in addition to the conventional Papanicolaou test, detection of viral antigens in cervical cells would be desirable by at least two reasons: (1) establishing risk population and (2) helping to solve the actiological background of the neoplastic changes of the cervix uteri.

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Letter to the Editor

A Proposal for Assessment of Hormone Sensitivity and Consequent Endocrine Therapy of Breast Cancer

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THE OESTRADIOL receptor content of breast cancer specimens is now widely used as an indicator for hormone sensitivity which is a prerequisite for endocrine therapy. Although positive correlations between receptor content and response to endocrine measures have been reported, a comparative evaluation of these data remains difficult. This is, to some extent, due to the different assay techniques and reference parameters employed, but probably more so to ovarian-independent fluctuations in the oestradiol receptor levels. We recently described such ovarian-independent fluctuations for several mammalian tissues [1] including human breast cancer: 1. An apparent circadian rhythm influences the uterine oestrogen receptor concentration of ovariectomised rats. 2. A seasonal variation was found in uteri of immature calves and ovariectomised pigs and in breast cancer biopsies from postmenopausal women. 3. A fluctuation with a periodicity of 9–12 days was observed in the uteri of ovariectomised/hypophysectomised rats. Circadian and seasonal fluctuations might be attributed to the release of adrenal steroids which are peripherally aromatized. In contrast, the fluctuating pattern of uterine oestrogen receptor concentration in ovariectomised/hypophysectomised rats points to a steroid-independent turnover of "receptor" [2].

The non-existence of "normal base-line" values considerably reduces the value placed on the quantitative oestrogen receptor assay as the sole indicator for hormone-sensitivity. Therefore, this assay should be complemented by further criteria. 1. The assessment of nuclear-bound oestradiol is both a logical choice and an easy task. The presence of the hormone would demonstrate that the receptor is still susceptible to steroid-facilitated conformational change, dimerisation and translocation into the nucleus. 2. Intact transcription-enhancing capability could be deduced from the presence of a specific transcription/translation product, such as the progestin receptor, the synthesis of which apparently depends on oestrogen receptor action [3].

Together, these 3 parameters should provide a reliable indication for hormone sensitivity, as demonstrated by the presence, in 10 normal human uteri, of substantial amounts of oestrogen receptor, oestradiol in the nuclear fraction and progestin receptor.

In mammary cancers, quite diverse patterns were observed: 3 out of 35 cancers so far investigated contained neither receptor nor nuclear oestradiol, indicating a failure in steroid binding, receptor "activation" and translocation. Six carcinomas contained oestrogen receptor and oestradiol in the nuclear fraction but no progestin receptor, pointing to a failure in the transcription-enhancing events. Two cancers, exceptionally, contained no oestrogen receptor, but both nuclear oestradiol and progestin receptor were present. This could be explained either by experimental error, or by the presence of trace (undetectable) amounts of oestradiol receptor, and underlines the importance of having more than one parameter for assessing hormone sensitivity. The remaining 24 cancers showed a pattern similar to that seen in normal human uterus.

Most of the postmenopausal cancers examined contained surprisingly high nuclear oestradiol concentrations. Since oestrogen production of the ovaries declines rapidly after menopause, this oestradiol must mainly orig-

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inate from peripherally aromatised adrenal precursors.

An ablative endocrine treatment of patients, whose mammary cancers fulfill all 3 criteria for hormone sensitivity, should therefore ideally comprise ovariectomy and adrenalectomy, since ovariectomy alone obviously does not secure hormone-withdrawal.

However, in view of our latest results, it must be doubted that even the complete withdrawal of hormones is fully effective. Besides the already mentioned turnover of the oestradiol receptor in the uteri of ovariectomised/hypophysectomised rats, the receptor content of purified nuclei isolated from uteri of chronically castrated pigs was found to be about 5 times higher than the corresponding oestradiol content. This discrepancy could be explained either by a separate release of oestradiol and receptor which had originally entered the nucleus jointly, or by a steroid-independent entrance of the "receptor", which in itself possesses transcription-enhancing activity. The latter possibility is the more probable one and consequently receptor-"poisoning" in addition to hormone withdrawal must be envisaged as a therapeutic requirement. The experiments of Lippmann *et al.* [4] on human mammary cancer cell lines, the growth rates of which are enhanced by oestradiol and decreased by antioestrogens, lend support to this concept.

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Perspectives in Cancer Research

Therapeutic Significance of Sex-Steroid Hormone Receptors in the Treatment of Breast Cancer*

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I. INTRODUCTION

SINCE the original observation by Beatson [1] who reported in 1896, 2 regressions of mammary carcinomas following ovariectomy, endocrine therapy of advanced breast cancer has been widely applied. Two main modalities are used to this effect: on one hand, endocrine surgery (ovariectomy, adrenalectomy or hypophysectomy) and radiocastration; on the other hand, administration of different steroid hormones or their antagonists. The remission rate obtained with these treatments varies somewhat but is in the neighbourhood of 30%. The problem of selecting patients who will respond favorably to therapy has always preoccupied the clinician, in order to avoid unnecessary interventions and loss of time which is sometimes irretrievable. Radiobiological measurements of urinary or plasma steroid hormones, steroid sulfatation by tumor extracts as well as response to hormone in organ cultures have been proposed as prognostic tests. The validity of such tests has, however, never been established. In contrast, the study of the mode of action of sex steroids hormones has introduced another selecting test which seems valuable for assessing the probability of responding to endocrine therapies.

The capacity to concentrate steroid hormones is a characteristic of their target organs (i.e. estrogens and progestins for uterus and vagina, androgens for prostate, testis). This phenomenon is due to the presence in the tissues of these organs of specific cytoplasmic steroid–hormone binding proteins. These proteins, called "receptors", are assumed to play an essential part in the mechanism of action of these hormones. After their entrance into the target tissue cells, steroid hormones bind to their own specific receptors and convert them to an "activated form" [2]. The activated steroid–receptor complexes are then translocated into the nucleus where they bind to the chromatin. This interaction results in a nuclear retention of the hormones. An immediate increase in the ability of the chromatin to bind ^{3}H-actinomycin D [3, 4] and to synthesize RNA [2] seems also to derive from the interaction. These changes in transcriptional properties are followed by increased protein synthesis. Although the functions of these various metabolic processes remain unsettled, it is likely that some of them are related to the growth and/or differentiating effects of the steroid hormones.

With regard to human breast cancer Folca *et al.* [5] observed in 1961 that the uptake of administered ^{3}H-hexestrol by cancer tissues was higher in 4 patients who responded to adrenalectomy than in 6 others who did not respond to the operation. On the basis of this observation, the presence of estrogen receptors (ER) was searched for in samples from primary and metastatic mammary tumors. Their presence was demonstrated around 1970 by Korenman and Dukes [6] as well as by Jensen *et al.* [7]. Furthermore, in a clinical study of patients with advanced breast cancers, Jensen *et al.* [7] reported that most patients with "ER-positive" tumor biopsies obtained an objective remission with adrenalectomy while those who were "ER-

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negative", usually failed to respond to therapy. These studies on biochemical-clinical correlations were repeated in several centers and extended to androgen (AR) [8, 9] and progesterone (PgR) [10] receptors as well as to other forms of endocrine treatments. It is the purpose of the forthcoming paragraphs to describe the various assays used for the detection of the receptors and to analyse the therapeutic significance of the receptors in the treatment of breast cancer.

II. METHODS FOR STEROID HORMONE RECEPTORS ASSAY IN NEOPLASTIC TISSUES

Numerous methods have been developed to measure with accuracy the receptors concentrations in neoplastic human mammary tissues [11]. Assays are usually restricted to cytoplasmic receptors which are measurable in extracts from fresh or frozen tissues.

Homogenization of the tissue specimen is the first step of the assay. The addition of a sulfhydryl reagent (β-mercaptoethanol or dithiothreitol) to the homogenization buffer increases the sensitivity of the method [12]. The homogenate is then centrifuged to obtain the cytosol fraction. The demonstration of labeled steroid specifically bound to this fraction permits the detection of receptors in the tissue samples; ^{3}H-estradiol-17β is used for the detection of ER, ^{3}H-dihydrotestosterone for AR, ^{3}H-R-5020* (a progestin) for PgR. R-5020 is usually used instead of progesterone because unlike the latter hormone, it does not bind to the plasma contaminant CBG [13–15]. Specific ^{3}H-steroid complexes may be distinguished from unspecific binding by one of the following two methods.

(i) Two cytosol fractions are incubated with nearly saturating amounts of ^{3}H-steroid. A large excess of unlabeled steroid is added to one fraction in order to distinguish specific from unspecific binding.

(ii) Aliquots of the cytosol fraction are incubated with increasing amounts of ^{3}H-steroids. The specificity of the binding reaction is determined by the graphical method of Scatchard [16].

After incubation, unbound steroid is removed to detect labeled steroid receptors complexes. Of the methods most frequently used, the *sucrose density gradient* has the unique advantage of evaluating the molecular shape of these complexes [7, 11]. In dilute buffer, without added salt they migrate as aggregates of which the sedimentation coefficients are close to 8 S. In some cases, 4 S complexes are also observed. In presence of high amounts of contaminating serum proteins in the cytosol fractions, the identification of these 4 S forms may be quite difficult because most contaminating serum binding proteins sediment in the 3–5 S region of the gradient. This difficulty does not exist with *agar-gel electrophoresis* [17] in which all specific tritiated molecular forms migrate as a single peak that can more easily be distinguished from the contaminating serum binding proteins. Both sucrose density gradient sedimentation and agar-gel electrophoresis require expensive equipment. This is not the case for the *dextran-coated charcoal* (*DCC*) method [18], which has the additional advantage of being easy, fast and more sensitive. This method, which is used in our laboratory [19], is based on an adsorption procedure: the addition of a dextran-coated charcoal suspension to the ^{3}H-steroid containing cytosol preparation rapidly removes most unbound and unspecifically bound labeled hormones while it has no significant effect on the amount of specifically bound radioactivity. *Protamine sulfate precipitation* [20] is another simple method which was recently introduced. It is based on the observation that receptors precipitated from the cytosols by protamine sulfate are still able to bind labeled steroids. It has the advantage of easily measuring total receptors binding sites, free as well as occupied by endogenous hormones. It should be stressed that, among all these methods, dextran-coated charcoal cannot be used for measuring AR because the charcoal treatment fails to remove ^{3}H-dihydrotestosterone bound to the plasma contaminant SHBG. In contrast, agar gel electrophoresis [21] and protamine sulfate precipitation [22] were shown to be especially accurate for the study of AR.

The binding capacity of the cytosols is generally expressed in fmole (10^{-15} mole) per mg *total* cytosol protein or *tissue* protein after correction for contaminating serum proteins. For assessing tissue protein, serum albumin is measured by a radioimmunodiffusion assay and the following formula is used [18]:

$$\text{tissue protein} = \text{total protein} - \text{serum albumin} \times \frac{100}{60}$$

*R-5020: 17, 21-dimethyl-19-nor-pregna-4, 9-diene-3, 20-dione.

III. OCCURRENCE AND CONCENTRATION OF SEX-STEROID HORMONE RECEPTORS IN HUMAN BREAST CANCERS

1. *Estrogen receptors*

In *female* breast cancers, the proportion of ER-positive tumors varies from laboratory to laboratory (range: ~35–85%). In some of them, including ours [23], the proportion steadily increased as the study progressed. Such progressive changes may probably be ascribed to technical improvements. Our present-day results indicate that about 85% of primary and metastatic breast cancers contain ER [24]. In the receptor-positive lesions, receptor concentrations were found to be extremely variable from tumor to tumor [23]. They varied over a wide range of values from about 5 to 2000 fmole/mg of tissue protein. Within this range the distribution of tumors appeared continuous with a progressive increase in proportion towards the lower values. Besides differences in sensitivity of methods, it is likely that the reported disagreements in the proportion of ER-positive tumors result from the criteria used to define positivity. Many investigators use, for that purpose, an arbitrary minimal concentration of binding sites. The continuous distribution of ER concentrations together with a very small proportion of "negative" tumors in our series is not in favor of the existence of two classes of breast cancers on the basis of *presence* or *absence* of ER. It is our feeling that most, if not all, do contain receptors although some in too low concentration to be detected by the current assays.

It is now established that the mean ER concentration is lower in women before than after the menopause [25]. Whether this difference is due to physiological changes or to partial saturation of receptors sites by endogenous estrogens is not established. Recent preliminary data suggest, however, that low concentrations of ER found in premenopausal patients cannot be explained by partial saturation of sites [26].

In *male* breast cancers, the presence of ER was also detected in primary and metastatic tumors. A recent report from our laboratory gives the more complete investigation on this matter [27]. ER were found in a large majority of primary (6/7) as well as in 1 out of 3 metastatic tumors. Receptor concentrations varied from tumor to tumor. Finally, no difference was detected between estrogen binding specificity of cytosol preparations from a male and a female breast cancer. All these data suggest a great similarity between estrogen receptivity of male and female mammary neoplastic tissues.

2. *Progesterone receptors*

The presence of PgR in some *female* breast cancers was first reported by Terenius [28] using ^{3}H-progesterone as labeled hormone. This observation was confirmed by Horwitz and McGuire [15] using the tritiated progestin R-5020 (see Section II). Later on, presence of PgR was reported in *male* breast cancer [24, 29].

In our laboratory, PgR was detected in 59% of primary and 47% of metastatic tumors [24]. These proportions are within the range reported by others (~35–60%) [30]. It is likely that the difference in proportions of receptor-positive lesions are partially due to the criteria used to define positivity. In contrast, there is a general agreement that the presence of PgR is essentially restricted to ER-positive cancers. Furthermore, data from our laboratory indicate that lesions positive for both ER and PgR are more frequent in primary than in metastatic lesions suggesting a loss of progesterone receptivity during the metastatic process [24].

Our investigations indicate that in PgR-positive cancers, receptor concentrations varied over a wide range of values from about 5 to 5000 fmole/mg tissue protein [24]. Within this range, the tumors were distributed in a continuous manner, with a progressive increase in proportion from the higher towards the lower values. This pattern was the same as for ER (see above). Furthermore, in cancers containing both ER and PgR, receptor concentrations were significantly correlated. Absence of PgR in ER-positive tumors was, however, associated with ER concentrations distributed along the whole scale. Similar observations were reported by other investigators [30].

Mean PgR concentration was found to be higher in pre- than in post-menopausal women. This difference might be explained by the higher level of circulating estrogens in the former patients.

3. *Androgen receptors*

Wagner *et al.* [31] first detected the presence of AR in some primary and metastatic *female* breast cancers. This observation was confirmed by Engelsman *et al.* [32] as well as by Maass *et al.* [33]. Whereas the two latter groups reported a quite low rate of receptor-positive tumors (~20%) Wagner and Jungblut [21] found AR in about half the cases. Differences in receptor assays seem unlikely to explain this discrepancy since all authors used the same

*Table 1. Relationship between presence of estrogen receptors and response to endocrine therapy**

ER	(No. remission/No. total) Therapy		
	Ablative surgery†	Steroid hormone administration‡	Other hormonally-related compounds (mainly antiestrogens)
+	59/107 (55%)	51/85 (60%)	10/23 (43%)
−	8/94 (8%)	7/82 (8%)	5/32 (16%)
Total	67/201 (33%)	58/167 (35%)	15/55 (27%)

*Data taken from McGuire *et al.* [25].
†Adrenalectomy, castration and hypophysectomy.
‡Androgens, estrogens and glucocorticoids.

technique (agar-gel electrophoresis). AR concentrations were found variable from tumor to tumor. On the average, they are largely lower than ER concentrations [21].

IV. ABSENCE OF RELATIONSHIP BETWEEN ESTROGEN RECEPTORS AND PATHOLOGICAL FINDINGS

Consistent correlation studies between sex-steroid hormones receptors and pathological findings are only available in the case of ER.

Most investigators found no correlation between the presence of ER in a tumor and its histological type [25]. Rosen *et al.* [34] reported, however, a higher frequency of ER-positive tumors in the invasive lobular carcinomas than in the typical ductal ones. The same authors also reported that receptors were less frequent in tumors with a prominent local lymphocyte reaction. Finally, no correlation was found between ER concentrations and the epithelial cell density of the tumors.

A well-established fact is that the presence as well as the concentrations of ER in a primary breast cancer bears no relationship with the presence or absence of invaded axillary nodes [25]. This suggests that the estrogen receptivity of a primary tumor is of little or no prognostic value since the axillary nodal status is the factor of greatest prognostic significance for survival [35].

V. SEX-STEROID RECEPTORS AS A GUIDE FOR ENDOCRINE THERAPY IN ADVANCED BREAST CANCER

1. *Estrogen receptors*

Qualitative assessment of estrogen receptors for endocrine therapy. In 1974, an international workshop was held for evaluating the relationship between presence of ER in tumor of patients with advanced breast cancer and response to endocrine therapy [25]. A total of 436 treatment trials from various centers were reviewed by two independent oncologists.§ It was found that 55% of the patients with ER-positive cancers responded to ablative endocrine procedures (Table 1). In the same way, 60% of the positive patients had a remission after administration of pharmacological doses of androgens, estrogens or glucocorticoids. A somewhat lower proportion (43%) was obtained after administration of a variety of other additive therapies including antiestrogens, aminogluthetimide, etc. On the other hand, in ER-negative patients, only 8–16% responded to these various treatments.

These results led to the conclusion that the detection of ER in advanced breast cancer was helpful in predicting response to endocrine therapies. Nevertheless, they were also disappointing because the predictive value of the receptors test was much weaker than in Jensen's initial report [7]. The question then arose why many ER-positive cases failed to be clinical responders.

Quantitative assessment of estrogen receptors for endocrine therapy. In a large series of 123 women, Jensen [36] observed that patients without an arbitrary minimal ER concentration had virtually no chance of responding to any kind of endocrine therapy. This minimal receptor concentration in the tumors appeared to be lower in pre- than in postmenopausal women. Above this minimal concentration, about 65% of the patients were found to respond to various endocrine therapies. In this range there was, however, no significant correlation between ER level and the likelihood of a remission. This

§Dr. M. E. Sears and G. C. Escher.

approach simply increased the proportion of responders in the arbitrary-defined ER-positive patients as compared to the whole ER-positive group.

Search for a quantitative relationship between the likelihood of obtaining a remission and ER concentration was performed at our Institute in a series of 34 patients using a linear logistic regression model [37]. Eleven additional clinical variables of possible relevance to response [35] were included in the analysis (age, menopausal status, postmenopausal yr, presence or absence of a free interval, duration of free interval, primary treatment vs secondary treatment, soft tissue involvement, bone involvement, lung involvement, liver and abdomen involvement, performance status). It was found that ER concentration was significantly correlated with the probability of obtaining a response to endocrine therapies and that among the 12 variables it was the most significant in this regard ($P=0.016$). Furthermore computation yielded a formula that served to construct a curve estimating the probability of response as a function of receptor concentrations (Fig. 1). Inclusion of 2 other significant variables (bone involvement, age) in addition to receptor concentration yielded a second curve which better fitted the experimental data.

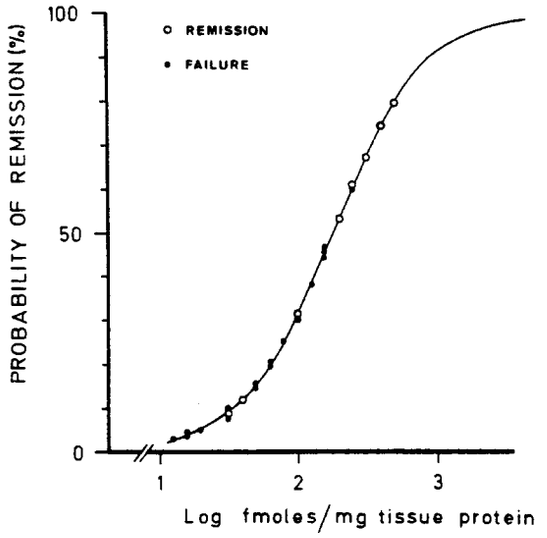

Fig. 1. Relationship between estrogen receptor concentrations and the probability of response to endocrine therapy. Experimental data were analysed by using the linear logistic regression model. The curve is a graphical representation of the statistical model.

Relationship between the sedimentation characteristics of estrogen receptors and response to endocrine therapy. At low ionic strength cytoplasmic ER sediments as a 8 S or/and 4 S components on linear sucrose density gradients (see Section II). Wittliff *et al.* [38] reported that patients having only 4 S binding components never respond to endocrine therapy. Although these data need confirmation, they suggest that the clinical usefulness of assays which do not distinguish sedimentation characteristics may be limited.

2. *Progesterone receptors*

Because in the uterus the synthesis of PgR is induced by estrogens and therefore requires an intact estrogen receptive machinery, Horwitz *et al.* [10] proposed that PgR might be an improved predictor of hormone dependency. This theory found support in the observation that PgR is absent in almost all ER-negative tumors but is present in about half the ER-positive ones (see Section III, 2).

With regard to clinical application, a preliminary correlation study by McGuire's group [10] suggested that the theory might be valid since, in a series of 9 patients, response occurred only in those with tumours containing both ER and PgR (3 cases). At a recent workshop on PgR [30] (Table 2), Bloom *et al.* [39] reported data in perfect agreement with those of this preliminary study. Present-day results from Horwitz and McGuire [40] indicated that among ER-positive tumors, remissions occurred in both Pg-positive and -negative cases with a higher rate of remission in the tumors containing both receptors. In contrast, in our own cases, no relationship was found between response and presence or concentration of PgR (Table 3). Remissions were, however, observed in cancers containing the highest levels of ER as in our previous study (Section V, 1). Differences in receptors assays might be responsible for the disagreement between the two former groups and ours. Further studies are needed to assess this point and to evaluate the exact predictive value of PgR. Nevertheless, it already appears that PgR is not an entirely reliable predictor of hormone dependency.

3. *Androgen receptors*

Only few data are available on the predictive value of AR for endocrine therapy. In a small series of patients, Maass [8] reported a higher rate of remissions in the receptor-positive (4/5) than in the receptor-negative cancers (6/20). In another small series, Persijn *et al.* [9] found a significant relationship between the presence of the receptors and the response to castration (AR-positive: 4/6; AR-negative 0/13; $P=0.004$). In contrast, they observed that the presence of AR had no significant predictive value for response to ethinylestradiol.

*Table 2. Relationship between presence of estrogen and progesterone receptors and response to endocrine therapy**

		(No. remission/No. total)		
ER	PgR	Horwitz and McGuire	Bloom *et al.*	Leclercq *et al.*
+	+	17/23 (74%)	6/7 (86%)	1/3 (33%)
+	−	5/14 (36%)	0/2 (0%)	1/3 (33%)
−	+	−	−	0/1 (0%)
−	−	0/8 (0%)	0/4 (0%)	−
Total		22/45 (49%)	6/13 (46%)	2/7 (29%)

*Data taken from McGuire, Raynaud and Baulieu [30].

Table 3. Estrogen and progesterone receptors concentration in relation to endocrine therapy†

Patient	ER (fmole/mg tissue protein)	PgR	Response
1	652	245	Remission
2	146	0	Remission
3	137	306	Failure
4	19	0	Failure
5	14	0	Failure
6	12	35	Failure
7	0	43	Failure

†Institut J. Bordet; data taken from Leclercq *et al.* [24].

VI. ANTIESTROGENS IN THE TREATMENT OF ADVANCED BREAST CANCER

A wide range of synthetic compounds are able to inhibit the growth-promoting effects of estrogens in the rodent's uterus. Although the mode of action of these *antiestrogens* is still largely unknown, evidence was reported indicating that some of them operate by interfering with the action of ER. These facts suggested that antiestrogens could inhibit growth of ER-positive breast cancers. As yet, the antitumor efficacy of 3 compounds *nafoxidine* (U-11, 100A)‡ (41, 42), *tamoxifen* (ICI-46, 474)§ (43–47) and *CI*-628‖ (43, 48) has been demonstrated in dimethylbenz(a)-anthracene-induced rat mammary tumors. Furthermore, in this experimental system, data were reported supporting the hypothesis that the action of antiestrogens is dependent upon the presence of ER in the tumors [43, 46]. Finally, in human breast cancer, *nafoxidine*, *tamoxifen* and *clomiphene*¶ proved to be therapeutically active.

In 1972, the E.O.R.T.C. Breast Cancer Cooperative Group reported a 35% objective remission rate in a series of 23 patients treated with *nafoxidine* [49]. This rate of remission is almost identical to that reported in another trial by Bloom and Boesen (37% in 48 patients) [50]. These exploratory studies were followed by a randomized trial comparing nafoxidine with ethinylestradiol [51]. Objective remissions occurred in 31% (15/49) and 14% (7/49) of the patients respectively. Although nafoxidine yielded a distinctly higher remission rate than ethinylestradiol, the difference was not significant at the 0.05 level ($\chi^2 = 2.872$; $0.05 < P < 0.10$). Recently, in a series of 24 patients, Sasaki *et al.* [52] reported that nafoxidine produces remissions only in ER-positive cancers, suggesting that its action in man is also mediated through these receptors. Furthermore, they found that it is effective only in the treatment of cutaneous metastases. Patients with lesions in other sites uniformly failed, for unknown reasons, to respond to therapy. Finally, nafoxidine was found to produce specific toxic reactions on skin and hair [50, 51] that limits its practical usefulness.

Tamoxifen and *clomiphene* have been reported to be therapeutically active without the major side-effects of nafoxidine. In 1971, Cole *et al.* [53] reported 10 objective remissions in 46 women treated with tamoxifen (22%). This rate was comparable to that obtained in two historical control groups, one treated with diethylstilbestrol (16 remissions out of 64 patients), the other with androstenediol (10 remissions out of 60 patients). The antitumor efficacy of the drug was confirmed by Ward [54] who observed objective tumor regression by more than 50% in

‡Nafoxidine (U-11, 100A) 1-[2-(p-[3, 4-dihydro-6 methoxy-2 phenyl-1 naphtyl] phenoxy) ethyl]-pyrrolidine hydrochloride.

§Tamoxifen (ICI-46,474) *trans*-isomer of 1-[p β-dimethylaminoethoxyphenyl-1, 2 diphenylbut-1-ene].

‖CI-628: 1-[2-(p-[α-p-methoxyphenyl-β-nitrostyryl] phenoxy)ethyl] pyrrolidine monocitrate.

¶Clomiphene: *cis*-isomer of 1-[p-(β-diethylaminoethoxy)phenyl]-1, 2-diphenyl-2-chloroethylene.

26 out of 68 patients. Clomiphene was investigated by Hecker *et al.* [55] in 50 women and 1 male. Objective remissions were found in 39% of patients (19 women, 1 male).

VII. STEROID HORMONES LINKED WITH CYTOXIC AGENTS IN THE TREATMENT OF ADVANCED BREAST CANCER

The potential interest of using steroid hormones or related compounds linked with cytotoxic agents is based on the principle that such molecules would specifically concentrate into the target tissues including breast cancer provided certain conditions are fulfilled. Among them, the drug should have a significant affinity for the receptors and should not be easily metabolized outside the target tissues into its two main moieties, cytotoxic agent and hormone. Subject to these provisions, the use of such molecules might increase the specificity of action and decrease the systemic toxicity of chemotherapy. Along this line, *estramustine phosphate* (*Estracyt*, Leo 299),* a phenolic N-bis (β chloroethyl carbamate of estradiol-17β phosphate) and *predmustine* (Leo 1031),† a chlorambucil ester of prednisolone, have been tested in clinical trials on patients with advanced breast cancer. The latter compound is not a sex-steroid hormone derivative, but its apparent therapeutic efficacy justifies its inclusion in this section.

In 1969, the E.O.R.T.C. Breast Cancer Cooperative Group [56] reported negative results in a series of 34 patients treated with *estramustine phosphate*. Only two remissions were obtained after daily i.v. administration of 60 mg of the drug. Since this rate of remission was lower than that expected from the use of either estrogen alone or cytotoxic agent, Estramustine phosphate was considered of no therapeutic value. This drug was recently studied *in vitro* in our laboratory [57]. Its affinity for ER was found extremely low. Moreover, it rapidly degraded into compounds of higher binding affinity in blood and in cytosol preparations from liver, uterus and mammary tumors. Evidence was given that estradiol-17β might be one of these degradation compounds. These observations may easily explain the therapeutic failure of estramustine phosphate in breast cancer.

The therapeutic efficacy of *predmustine* was investigated by Könyves *et al.* [58] in a series of 15 patients. After a daily oral administration of 20–100 mg of the compound, 2 complete and 5 partial remissions were recorded. Whether these remissions were induced by the drug itself or prednisolone and/or chlorambucil produced from its degradation, is unknown.

Other molecules are available [47, 59, 60]. Some of them are currently subjected to *in vitro* studies [47, 61] and animal experimentation in our laboratory.

VIII. CONCLUSIONS

The demonstration of the presence of sex-steroid hormone receptors in human breast cancer has fostered progresses in the therapy of the disease. First, the presence of ER, PgR and AR in tumor biopsies proved helpful in predicting the probability of a patient responding to endocrine therapy. Secondly, they allowed a legitimate hope of obtaining highly efficient and specific new treatments based on the use of drugs interfering with the mode of action of these receptors.

With regard to the first point, although the results are encouraging, it is already known that some cancers, even with high amounts of receptors, are clinically refractory to endocrine treatments. The reason for these therapeutic failures is unknown. Alterations in the receptor machinery (Section I) were proposed to be responsible. Thus, lack of transfer of ER from the cytosol to the nucleus [62, 63] and deficiency in ER-induced RNA synthesis [64] have been described in experimental models. These considerations are probably undue oversimplifications since tumors regress after a variety of endocrine treatments not necessarily involving steroid-related mechanisms. In this context, preliminary data suggest that tumors might be composed of several populations of cells in respect to sex-steroid receptors [65, 66]. According to this multiclonal theory, therapeutic failures may be due to selective hormone-dependent cell kill leading to preponderant growth of hormone-independent cells. Finally, it is obvious that steroid receptivity is only a part of a complex system regulating tumor growth in which polypeptide hormones could also play key roles. Thus, evidence was reported suggesting that prolactine controls the level of ER in dimethylbenz(a)anthracene induced rat mammary tumors and thereby modulates estrogen receptivity [67–69]. Refinements in sex-steroid

*Estramustine phosphate (Estracyt, Leo 299): estradiol-3N [bis-3(2 chloroethyl)carbamate-17β-dihydrogenophosphate.

†Predmustine (Leo 1031): pregna-1, 4-diene-3, 20-dione, 11β, 17α, 21-trihydroxy-21-4-p [bis(2 chloroethyl)amino] phenyl-butyrate.

and polypeptide hormone receptor assays seems therefore to be needed to improve knowledge in this matter and thereby increase the accuracy of testing hormone dependency. Unfortunately, such sophisticated assays will not be very convenient for use in clinical laboratories so that search for simple techniques should also be developed.

The clinician is now facing an alternative. Either he decides to follow a traditional therapeutic approach, i.e. endocrine treatment followed as a last resort by cytotoxic chemotherapy. If he selects this option, receptor assays may serve as a guide to his initial decision: in such a case, ER concentration will inform him on the probability of response to endocrine treatments (Fig. 1). Additional assays of PgR and AR will probably improve the predictive value of the test. Nevertheless, it remains that endocrine treatments will never exceed a success rate of 30%. The clinician may therefore adopt a distinctly different approach. Knowing that most breast cancers are hormone receptive at least to some extent and thereby more or less hormone-dependent, he should resort to endocrine treatments in *all* cases. He does not expect to *recognize* objective remissions in each, but he may assume that some beneficial effect has nevertheless been obtained. In this perspective, endocrine treatment should be complemented by cytotoxic chemotherapy in a majority of cases. Search is needed to define the optimal means of combining these two modalities.

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In Vitro Arming and Blocking Activity of Sera from BALB/c Mice Bearing a Spontaneous Transplantable Adenocarcinoma*

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Abstract—*The kinetics of the immune response of BALB/c mice to a syngeneic spontaneous adenocarcinoma (ADK-1t) were analyzed in vitro during the various stages of tumor growth. Cell-mediated cytotoxicity, as measured by a colony inhibition assay, was first detected 5 days after tumor inoculation, reached a peak at about 13 days, and then declined to low but significant levels by day 21.*

A peak of complement-dependent serum cytotoxicity was observed at 10 days when the tumor size was about 5 mm. Moreover, sera from animals bearing tumors of 1.5–10 mm dia were able to arm lymphocytes from normal BALB/c mice and enhance the cytotoxic activity of lymphocytes from tumor-bearing mice. On the other hand, no blocking activity was observed when the ADK-1t target cells were preincubated with the same sera. When cytotoxic lymphocytes were pretreated with sera from mice bearing tumors larger than 10 mm in size, inhibition of cell-mediated cytotoxicity was observed.

These findings show that the interaction of serum factors on cellular reactivity is of a complex, evolving nature, directly related to the tumor size.

INTRODUCTION

SPECIFIC cellular cytotoxicity against autochthonous or syngeneic tumors may be demonstrated in human patients and tumor-bearing animals by different *in vitro* assays. However, sera from patients and animals bearing tumors contain factors that strongly modify cellular reactivity. "Blocking factors" have been demonstrated in several host-tumor systems [for a review see 1]. The blocking activity seems to be mediated by 7S immunoglobulins [2], antigens shed from cell membrane [3, 4] or tumor-specific antigen–antibody complexes [5, 8]. On the other hand, "potentiating" [9] and "arming" [10, 11] serum activities have also been described.

However, the temporal evolution of the cellular reactivity and the influence of humoral factors have been studied mostly in particular systems, i.e. virus-induced tumors or in chemically induced neoplasms of particular antigenicity [12, 15].

In the present studies we analyzed the kinetics of the immune response of BALB/c mice against a syngeneic spontaneous mammary adenocarcinoma. Moreover, we defined the interaction of humoral factors from tumor-bearing animals with the cell-mediated cytotoxicity during the tumor growth.

MATERIAL AND METHODS

Mice and tumors

Inbred BALB/c mice, 6–8 weeks old, from our colony, were used. The syngeneic tumor studied was a mammary adenocarcinoma (ADK-1t) that arose spontaneously in our BALB/c colony. It was maintained for 20 generations by serial s.c. inoculations of 1×10^6 living cells, suspended in 0.2 ml of Hanks' basal salt solution (HBSS). The injection site was palpated for the presence of a tumor every two days and growth rates were measured with a caliper. Two sarcomas (Sa I and Sa II) arisen spontaneously in our colony were used as specificity controls.

Target cell cultures

ADK-1t, Sa I and Sa II monolayers were obtained *in vitro* as previously described in detail

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[16]. After treatment of the excised tumors with a 0.25% trypsin solution supplemented with 1000 U of deoxyribonuclease/ml (DNase I, Sigma) the cells were washed twice, layered on 3 ml of Lymphoprep (Nyegaard & Co., AS., Oslo, Norway) and centrifuged at $400 \times g$ for 40 min 4°C. The cells were subcultured *in vitro* for 2–3 passages before use. The cell lines obtained were constantly tested for tumorigenicity.

One hundred percent of syngeneic animals injected s.c. with 5×10^5 cultured tumor cells developed tumors resembling the primary tumors in their growth pattern and histological characteristics.

Sera from immune and tumor-bearing mice

Different groups of tumor-bearing mice, injected with 1×10^6 ADK-1t cells, were bled on the 3rd, 5th, 8th, 10th, 13th, 15th, 21st, 26th and 34th day after the transplant. ADK-1t hyperimmune sera were obtained from BALB/c mice injected 4 times at weekly intervals with 5×10^5 tumor cells pretreated with 40 μg/ml of mitomycin C (Sigma, St. Louis, Mo., USA) for 45 min at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. All the sera were heat inactivated at 56°C for 30 min and stored in separate vials at −20°C until required. They were diluted 1:5 with Eagle's MEM and filtered through 0.22 μ Millipore filters immediately before use.

Lymphocytes

Lymphocytes from normal and tumor-bearing mice were obtained from cervical, axillary and mesenteric lymph nodes, minced with forceps, and passed through a 100-gauge stainless-steel screen. The cells were washed twice in HBSS, suspended in Eagle's MEM supplemented with 15% fetal calf serum (FCS), and incubated 18 hr in tissue culture dishes (No. 3001: Falcon Plastics, Oxnard, Calif., U.S.A.) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air as described by de Landazuri and Herberman [17]. Then the nonadherent cells were collected, washed twice in HBSS, and suspended in Eagle's MEM at a concentration of 15×10^6 viable cells/ml. Cellular viability (determined by Trypan blue exclusion) was greater than 90% in all experiments.

Lymphocyte cytotoxicity

The colony inhibition (CI) test was performed as described by Hellström [18]. The CI test and the complement-dependent cytotoxicity assay (see below) were performed at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, using Eagle's MEM supplemented with 10% FCS. Eight hundred-thousand cultured ADK-1t, Sa I and Sa II tumor cells were seeded into each well (35 mm ϕ) of macroplates (Funke–Gerber, Munich, W. Germany). After 18 hr the medium was removed and 7.5×10^6 lymphoid cells in 0.5 ml of medium were added to the effector: target cell ratio of 10,000:1. After 45 min incubation, 2 ml of fresh medium were added. The plates were then incubated for 3 days, after which nonadhering cells were removed by repeated washing in HBSS. The cytotoxic assays testing the influence of sera from normal, immune or tumor-bearing mice on the cell-mediated immune response were performed in two ways. First, tumor target cells were incubated for 60 min at 37°C with 0.2 ml of 1:5 diluted sera and then washed twice before the addition of lymphoid cells. Second, lymphocytes (6×10^7) were incubated with 0.2 ml of 1:5 diluted sera for 60 min at 37°C, washed twice and then added to the target cells. After 3 days incubation the cells were fixed, stained with Giemsa, and the colonies were counted under low-power microscopy. Specificity controls were always performed on Sa I and Sa II target cells. Six macrowells were always set from each combination tested. The percentage of cytotoxicity obtained in the different test combinations was quantitated as follows:

$$\% \text{ destruction} = \frac{(\text{mean number of colonies in control conditions} - \text{mean number of colonies in test conditions})}{\text{mean number of colonies in control conditions}} \times 100$$

$$\% \text{ blocking} = \frac{(\% \text{ destruction with normal serum} - \% \text{ destruction with test sera})}{\% \text{ destruction with normal serum}}$$

Cytotoxic antibody test

The cytotoxic activity of sera from immune or tumor-bearing mice was assayed as described by Hellström [18] with some modifications. One hundred and fifty to two hundred cultured tumor cells in 0.2 ml of medium were distributed in 96 well microplates II (No. 3040; Falcon Plastics). After 18 hr the medium was removed and 0.1 ml of 1:5 diluted test or control sera was added.

After 45 min the sera were removed and 0.1 ml of fresh guinea pig serum as complement (C) source absorbed with mouse red cells was added

to each plate, except to the controls. The plates were reincubated 24 hr and washed twice with HBSS. Cell viability was determined by adding to each culture 0.1 ml of 1:500 dilution of fluorescein diacetate (FDA, Sigma) in HBSS [19]. After 10 min at room temperature, the FDA was removed, the wells gently rinsed 3 times and observed with a fluorescent microscope (Leitz Ortholux, Leitz, W. Germany) equipped with a vertical illuminator, BG 38 and BG 12 exciting filters and a TK 580 suppression filter. The brightly fluorescent living cells were counted and the percentage of cytotoxicity was quantitated as follows:

$$\%\ \text{cytotoxicity} = 100 - \frac{\text{number of cells in presence of test serum} + \text{C}}{\text{number of cells in presence of control serum} + \text{C}} \times 100$$

Immunofluorescence test

To perform the indirect immunofluorescence experiments viable ADK-1t cells were distributed on macroplates (Funke–Gerber).

After 24 hr of incubation to allow attachment, the cells were washed 3 times with HBSS and incubated for 30 min at 4°C with 1:5 diluted serum (5th, 10th, 21st and 34th day after the transplant). After 3 washings with HBSS solution, 0.1 ml of various dilutions of fluorescein-conjugated goat antiserum to mouse gammaglobulin (Hyland Laboratories, Los Angeles, Calif., USA) was added to each well and the cells were incubated for 30 min at 4°C. They were then washed 3 times with HBSS solution and observed with a Leitz microscope. The highest goat antiserum dilution to give fluorescence was selected as the serum titer.

RESULTS

Temporal evolution of cellular cytotoxicity

The cytotoxic response of lymphocytes removed at various times after tumor injection was tested *in vitro* against ADK-1t, Sa I and Sa II cultured target cells (Fig. 1).

A cytotoxic activity of about 25% against ADK-1t cells was first detected 5 days after tumor inoculation, when the neoplastic mass was not yet palpable, reached its maximum at 2 weeks when the mass was about 11 mm dia, and then gradually decreased to low but significant levels. By contrast, no activity was observed on Sa I and Sa II target cells.

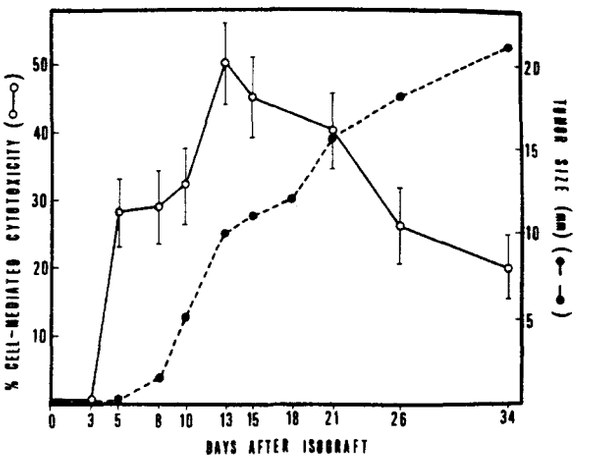

Fig. 1. Temporal evolution of cell-mediated cytotoxicity against ADK-1t cells. Lymphocytes were taken from tumor-bearing mice at progressive periods during tumor growth. Each point represents the mean of 6 separate determinations. Vertical bars: ± S.E.

Temporal development of anti-ADK-1t antibody

Sera from ADK-1t-bearing mice were collected at progressive times after tumor injection and tested for antibody activity by indirect immunofluorescence and C-dependent cytotoxicity (Fig. 2). Fluorescent antibodies against ADK-1t were first detected 10 days after challenge with a titer of 1:40. Later sera resulted in lower titers. In comparison, isoimmune serum produced fluorescence down to a dilution of 1:160. An increase of C-dependent cytotoxicity against ADK-1t cells was found at 8 days, with a tumor size of less than 3 mm dia, reached a peak of 20% two days later and decreased thereafter.

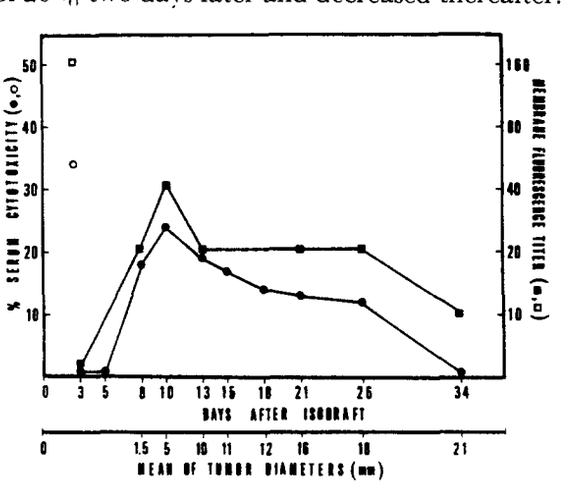

Fig. 2. Temporal evolution of complement-dependent cytotoxicity and membrane fluorescence titer of sera from ADK-1t-bearing and immune mice against ADK-1t cells. ●, ■: sera collected from tumor-bearing animals at progressive times during tumor growth and tested in presence of guinea pig complement ($P < 0.01$ from 8th to 13th day) or mouse gammaglobulin. ○, □: sera from animals immunized four times with ADK-1t mitomycin-C treated cells, tested in presence of fluorescein-conjugated goat antiserum to mouse gammaglobulin. The final dilution of all sera was 1:5. Each point represents the mean of 6 separate determinations.

A value of 34% was observed with isoimmune sera at the same dilution. In contrast, no cytotoxicity was observed on Sa I and Sa II control cells.

Temporal development of arming and potentiating activities of tumor-bearer sera

Lymphocytes from normal animals or from animals injected 2 weeks earlier with ADK-1t were added to target cells previously preincubated with different heat-inactivated sera as shown in Fig. 3. Normal lymphocytes were strongly activated when the target cells were pretreated with early sera obtained from tumor-bearing animals. This arming activity was first evident when ADK-1t cells were preincubated with sera obtained when no tumor mass was still evident, reached a peak with sera from animals bearing tumors of 2–5 mm dia, and decreased progressively during the tumor growth. Neither a potentiating nor a blocking activity was observed when cytotoxic lymphocytes were employed as effector cells. However, the preincubation of target cells with isoimmune antisera strongly armed normal lymphocytes and potentiated cytotoxic lymphocytes. The heat-inactivated sera alone did not show any cytotoxicity to target cells.

Moreover, control experiments with Sa I and Sa II target cells showed that the arming and potentiating activity of the sera were specific for ADK-1t cells only.

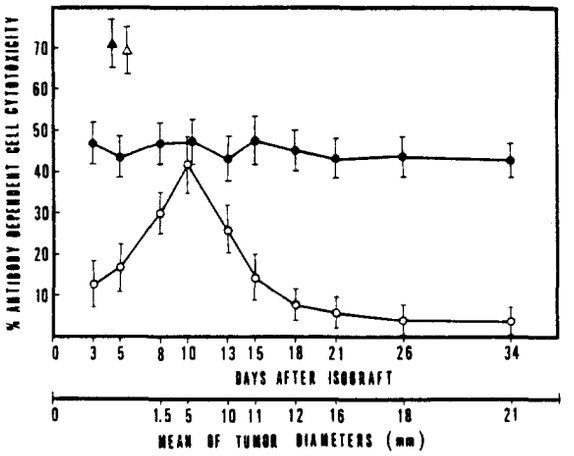

Fig. 3. Temporal evolution of arming activity of sera from ADK-1t-bearing mice against ADK-1t cells detected by CI. After preincubation of target cells with isoimmune sera and sera collected at progressive periods during tumor growth, normal or cytotoxic lymphocytes were added. ○: normal lymphocyte arming activity of sera from animals bearing progressively growing tumors. △: Normal lymphocyte arming activity of isoimmune sera. ●: cytotoxic lymphocyte activity in presence of sera from animals bearing progressively growing tumors. No potentiating or blocking effect was evident. ▲: cytotoxic lymphocyte potentiating activity of isoimmune sera. Each point represents the mean of 6 separate determinations. Vertical bars: ±S.E.

Temporal development of blocking activity of tumor-bearer sera

Immune lymphocytes from mice transplanted 2 weeks before with ADK-1t were preincubated with sera from ADK-1t-bearing mice removed at progressive periods after tumor injection and tested on untreated target cells (Fig. 4).

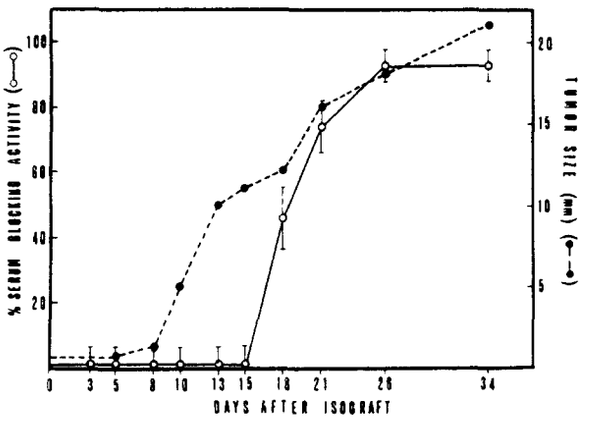

Fig. 4. Temporal evolution of blocking activity of sera from ADK-1t-bearing mice detected by CI. Cytotoxic lymphocytes were preincubated with sera collected at progressive times during tumor growth. Each point represents the mean of 6 separate determinations. Vertical bars: ±S.E.

When the immune lymphocytes were preincubated with sera from animals with tumors not yet palpable or with tumors not exceeding 10 mm dia, no blocking activity was observed. However, when the effector cells were preincubated with sera from animals with larger tumors, a sudden and rapidly increasing inhibition of cytotoxic activity was evident.

It should be noted that the serum blocking activity observed appears not to be directly related to the time factor but to the tumor size. When tumors were obtained either later or earlier than in the standard protocol by injecting different tumor cell numbers as previously described [20], the serum inhibitory activity always correlated with the tumor size and not with the time (Table 1).

Table 1. Correlation between tumor size and serum blocking activity

Number of ADK-1t cells injected	Days after injection	Tumor size (mm)	Serum blocking activity %
1×10^7	7	12±2	38±8
1×10^7	10	18±1.5	86±4
1×10^5	20	7±3	−2±5
1×10^5	29	15±3	75±7

The preincubation of normal lymphocytes with isoimmune, early or late sera did not stimulate cytotoxicity.

DISCUSSION

The ADK-1t mammary adenocarcinoma used in these studies is a tumor of moderate antigenicity [21] which presents several antigenic and structural membrane features that are not to be found in the normal cells [22, 23]. Previous work has also indicated that the growth rate of this tumor is influenced by spontaneous or artificially produced changes in the host immune reactivity, suggesting that its growth in a syngeneic host is hindered by a self-induced immunological mechanism [20].

In this host-tumor system, a marked level of cellular and humoral immunity was observed during the earlier phases of tumor growth before the appearance of a palpable neoplastic mass. Cytotoxic antibodies can be detected 8 days after the inoculation of 1×10^6 ADK-1t living cells. Similarly the cellular reactivity increased progressively and reached a peak about 2 weeks after inoculation in mice with a tumor size of 10 mm dia and then progressively decreased. It must be noted that animals with greater tumor diameters still had cytotoxic effector cells although at low, but significant, levels. These findings are in contrast to the data of Le Francois *et al.* [24] who found that cell-mediated cytotoxicity disappeared entirely after 21 days in a similar mammary tumor system. The discrepancy between our and Le Francois's findings could be ascribed to the preincubation of effector cells for 18 hr before cytotoxicity testing.

This development and decline of cellular cytotoxicity are similar to those reported in several host-tumor combinations in which a cytotoxic peak was detectable about 15 days after challenge [9, 14, 15, 25, 26, 27].

While in some host-tumor systems the evolution of cellular reactivity seems directly related to tumor size [25, 28], it is quite possible that this peak of cellular reactivity is mainly related to a general pattern of cytotoxic lymphocyte activation. In fact, a peak of cytotoxicity can be seen in several animal systems about 10 days after immunization with allogeneic or heterologous cells or with non cellular antigens [29].

In contrast, the different interfering serum activities sequentially observed in our ADK-1t-BALB/c system appear to be directly related to tumor size. A normal lymphocyte arming activity was detectable only with early sera from mice with a tumor size of 10 mm. Beyond this critical size there was a rapid decrease of the sera arming ability. At the same time a blocking activity of cytotoxic lymphocytes emerged and sharply increased. Therefore, the tumor size and not the time factor appears to be the critical determinant for the switch from the arming to the blocking activities. The appearance of the blocking activity may be related to an increase of free or antibody-complexed membrane antigens shed by growing tumors [7, 8, 30]. This may gradually become excessive and so block the arming antibody activity [31]. Relatively small variations in tumor size can therefore determine a rapid inversion of serum activity. A similar blocking activity *in vitro* within a narrow range of antigen and antibody concentrations has been reported by Baldwin [6].

The failure to affect the ADK-1t growth rate *in vivo* by passive administration of blocking serum and/or solubilized membrane antigens [32] may also be related to the use of doses lying outside a critical concentration range.

In agreement with Blair and Lane [33], the method used to demonstrate *in vitro* serum activity may well be of importance. Not a blocking but an arming activity is detectable after preincubating target cells with sera from ADK-1t-bearing mice. Otherwise, not a recruitment of normal lymphocytes but a block of cytotoxic lymphocytes is obtained after preincubating directly lymphoid cells with sera from tumor-bearing mice.

In conclusion, these data indicate that the tumor size-dependent evolution of serum activities may play a central role in modulating the magnitude of the cellular reactivity to ADK-1t detectable *in vitro* by colony inhibition technique. After an initial arming and potentiating influence the latter sera switch off the specific cytotoxicity of lymphoid cells. In contrast, the strong arming and potentiating activities of isoimmune sera appear to be an important cooperative factor *in vitro*. The ability of isoimmune sera to increase the initial host resistance and to overcome the blocking serum activity *in vivo* is presently under investigation.

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Influence of Altered Lysosomal Enzyme Activities on the Regression of DMBA-Induced Rat Mammary Tumours*

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Abstract—*The activities of two lysosomal acid hydrolases and one pyridine nucleotide-linked dehydrogenase were studied in relation to the regression of the hormone dependent DMBA-induced rat mammary tumour. Tumour regression was characterized by an elevated activity of p-nitrophenylphosphatase and β-glucuronidase whereas the activity of glucose-6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase decreased. Administration of cycloheximide to ovariectomized animals reduced [^3H]-leucine incorporation into tumour proteins, inhibited the elevated activities of the lysosomal enzymes studied and reduced the decrease in tumour mass resulting from the operation.*

INTRODUCTION

IN RECENT years there has been a great deal of evidence to indicate that lysosomal enzyme levels are increased during the processes of tissue disruption, degradation or reabsorption [1, 2]. Several reports have linked the activity of lysosomal enzymes to changes which take place during tumour regression [3–7]. The precise relationship between these parameters has not, however, been established.

The present study has examined early changes in the activity of various lysosomal enzymes during the enforced regression of dimethylbenzanthracene-induced mammary tumours under different experimental conditions.

MATERIAL AND METHODS

Animals

Mammary tumours were induced in virgin female Sprague–Dawley rats (50 ± 2 days old) by intubation with a single dose of 20 mg dimethylbenzanthracene (DMBA) in 1 ml sesame oil. Animals were housed in groups of 4 and fed diet and water *ad libitum*. After 5 weeks, the rats were palpated for tumours at weekly intervals and the size recorded as the mean of two perpendicular diameters, one measured across the greatest width. No tumour was used in experimentation which did not attain an approximate size of 20 mm mean dia in the 23-week period following carcinogen administration.

Chemicals

DMBA and cycloheximide were purchased from Sigma Chemical Company Ltd., London. [L-4,5-^{3}H] Leucine (specific radioactivity 50 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. Tissue culture media 199 was purchased from Flow Laboratories Ltd., Ayr, Scotland.

METHODS

Removal of tissue biopsies. Small biopsies of tumour tissue were removed aseptically, under ether anaesthesia using a sterile trocar.

Experimental. Four experiments were undertaken:

(i) Homogeneity of tumour samples. Fifteen samples (*ca.* 50 mg each) from a normal, growing tumour, and also from a tumour removed from a 7 day ovariectomized animal, were subjected to replicate analysis with respect to enzyme content and [^{3}H]-leucine uptake.

(ii) Effect of ovariectomy on enzyme levels. Eight tumours were biopsied daily for 5 days and then on days 7, 14 and 21 after operation. Approximately 50 mg of tissue was removed with

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each biopsy. The biopsy removed at day 7 was sectioned and classified histologically as having characteristics indicative of either tumour growth or regression [7].

(iii) Effect of ovariectomy and cycloheximide treatment on enzyme levels and protein synthesis. Groups of animals were ovariectomized and given a single intramuscular injection of cycloheximide (0.5 mg/kg body weight, in 0.15 M NaCl) within 10 min of the operation. Control animals underwent the sham operation. Tumour tissue was biopsied at the time of the operation (prior to cycloheximide treatment) and on 5 subsequent days. Approximately 100 mg of tissue was removed with each biopsy and examined for changes in enzyme activity and [^{3}H]-leucine uptake.

(iv) Effect of ovariectomy and cycloheximide treatment on subsequent growth patterns of tumours. Groups of animals were ovariectomized and given a single intramuscular injection of cycloheximide (0.5 mg/kg body weight in 0.15 M NaCl) within 10 min of the operation. Control animals underwent the sham operation. Tumour size was measured weekly as the mean of two diameters. Biopsy samples were taken on day 7 from both groups and the tissue histologically classified as "growing" or "regressing".

Incorporation of [^{3}H]-leucine into tumour protein. Slices of mammary tumour (approximately 1 mm × 5 mm, 20 mg) were washed in 0.15 M NaCl and incubated for 4 hr at 37°C with 2 μCi [^{3}H]-leucine/ml tissue culture medium 199 (1 ml). The tissue was then washed twice in 0.15 M NaCl. All subsequent procedures were carried out at 4°C. Tissue slices were homogenized in 2 ml 50 mM Tris–HCl buffer pH 7.4, containing 1 mM EDTA, using a Sorvall-Omnimixer (5 × 15 sec at full speed with 1 min intervals. A sample of homogenate (0.5 ml) was stored at −20°C for enzyme and protein estimations and the remaining homogenate was mixed with an equal volume of a solution containing bovine serum albumin (1 mg) and leucine (0.1 mg). After 2 min, an equal volume of 10% (w/v) TCA was added. The contents of the tube were mixed and centrifuged at 1000 ***g*** for 15 min at 4°C. The pellet was separated from the supernatant and washed twice in ice-cold 5% (w/v) TCA. Washings and the original supernatant, containing TCA soluble radioactivity, were stored at 4°C. The pellet was then hydrolyzed in 0.4 M perchloric acid (PCA) (2 ml) for 60 min at 80°C, centrifuged, the supernatant collected, and the washing procedure repeated. The supernatants were combined. Aliquots (0.5 ml) of this supernatant and also of the TCA soluble fraction were added to 10 ml scintillator fluid (1 l. toluene containing 4 g, 2,5-diphenyloxazole, 0.1 g 1,4-di [2-(5-phenyloxazolyl)] benzene) and radioactivity measured using a Nuclear Chicago Mark 1 Scintillation counter. Results were expressed as counts/min/mg protein.

Enzyme assays

The procedures for the estimation of *p*-nitrophenylphosphatase and β-glucurondase activity, together with the method of protein determination, have been previously described [6]. The combined activity of glucose-6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase was assayed at 25°C by the method of Kornberg and Horecker [8]. One unit of enzyme activity was defined as the amount of enzyme which will convert 1 μmole of substrate per min at its optimal conditions.

RESULTS

Tumour homogeneity with regard to biochemical characteristics

A major consideration in studies involving the removal of sequential samples from one tumour must be the homogeneity of the tissue with respect to the end point investigated. Analysis of acid hydrolase and pyridine nucleotide-linked dehydrogenase activities (units/g protein) in 15 tissue samples (approximately 50 mg each) removed from a growing tumour indicated that all values were within 5% of the mean. A similar analysis of a tumour removed 7 days post-ovariectomy showed an 8% variation.

Effect of ovariectomy on enzyme activities of mammary tumours

Of the 8 tumours studied, 2 were histologically classified as "growing" at day 7. These showed no variations in enzyme activity over the subsequent 21-day period (Fig. 1). Marked changes were, however, observed in the 6 "regressing" tumours. These tumours showed elevated *p*-nitrophenylphosphatase and β-glucuronidase activities, with maxima at day 5. The enzymes have been previously shown to be associated with a sedimentable fraction following differential centrifugation at 330,000 × ***g*** min and display the phenomena of structural latency [9]. These features are characteristic of lysosomal acid hydrolases in other tissues [10–12]. The non-lysosomal enzymes, glucose-6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase slowly declined in activity over the 21 day experimental period (Fig. 1).

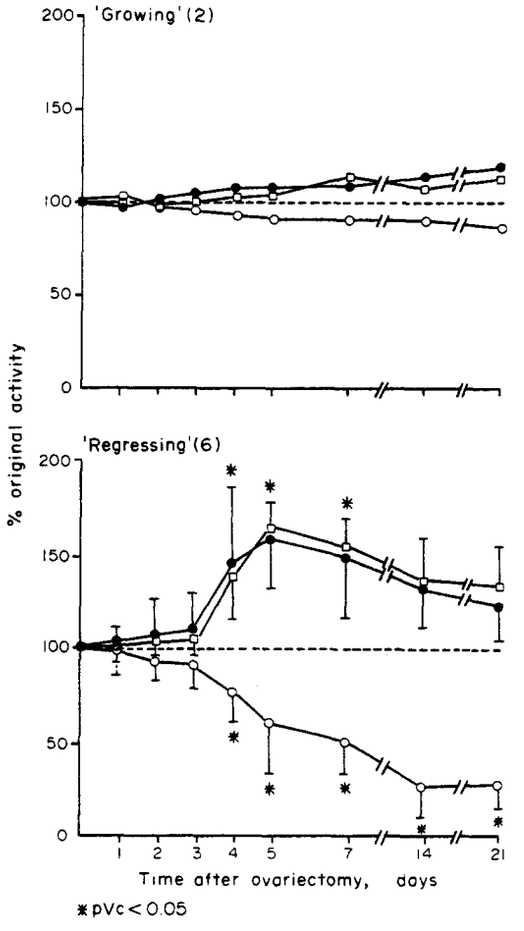

Fig. 1. Effect of ovariectomy on enzyme activities of mammary tumours. Tumour tissue was biopsied daily for 5 days *and then on* days 7, 14 *and* 21. *Tumours were classified histologically at* day 7 *as "growing" or "regressing". Results are expressed as a percentage of the original activity present within the tissue ± S.D. Activity was determined as units/g protein. Figures given in parenthesis indicate the number of tumours per group. p-nitrophenylphosphatase, ●——●, β-glucuronidase, □——□, glucose-6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase, ○——○. pVc (value determined at time 0) < 0.05 (P values were estimated by an analysis of variance with 4 replicate estimations).*

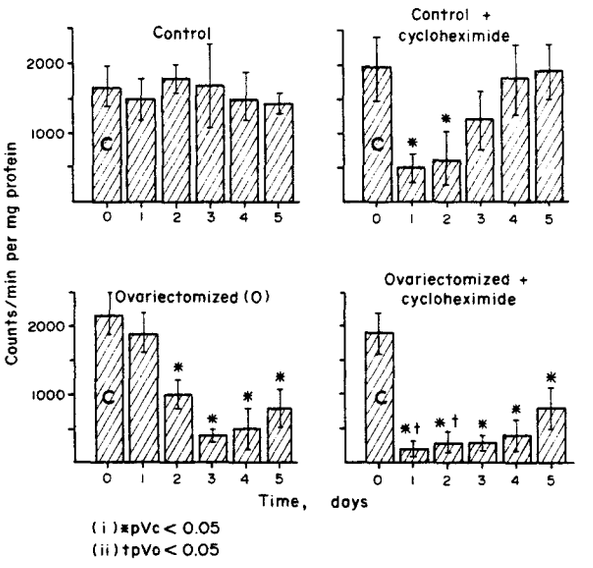

Fig. 2. Effect of ovariectomy and cycloheximide treatment on the incorporation of [^3H]-leucine into tumour protein. Groups of animals were ovariectomized and given a single i.m. injection of cycloheximide (0.5 mg/kg *body weight) within* 10 min *of the operation. Control animals were sham operated. Tumour tissue was biopsied at the time of the operation (prior to cycloheximide treatment) and on* 5 *subsequent days. Slices of tissue were incubated with [^3H]-leucine. Results show the mean of* 5 *tumours per group and are expressed as counts/min/mg protein ± S.D. Statistics (Students 't'-test for independent means) were performed by comparison of* (i) *the incorporation data determined on* days 1–5 *with the values determined at time* 0 (*$pVc < 0.05$) *and* (ii) *the incorporation data determined on corresponding days in ovariectomized animals with and without cycloheximide* (†, $pVO < 0.05$).

Effect of ovariectomy and cycloheximide treatment on the incorporation of [^3H]-leucine into tumour protein and on enzyme levels

[^{3}H]-Leucine incorporation into DMBA-induced rat mammary tumour protein was significantly reduced in tumours by day 2 after ovariectomy (Fig. 2). The levels of protein synthesis remained low during the remaining experimental period. Cycloheximide (0.5 mg/kg body weight) administered 10 min after ovariectomy further reduced [^{3}H]-leucine uptake into tumour protein as measured on day 1 or 2. Administration of cycloheximide to sham operated animals resulted in a marked decrease in the level of protein synthesis measured on days 1 and 2. The effectiveness of cycloheximide treatment was not however maintained over the remaining experimental period when levels returned to pre-injection values. The radioactivity found in the TCA soluble fraction was unaltered by any of the treatments.

Figure 3 presents data obtained from measurements of enzyme activity in these tumours. Cycloheximide treatment inhibited the ovariectomised-induced elevation of *p*-nitrophenyphosphatase and β-glucuronidase but did not affect the decrease in glucose-6-phosphate dehydrogenase/6-phosphogluconate dehydrogenase activity. Administration of cycloheximide to sham operated animals had no appreciable effect on any of the enzymes studied.

Effect of ovariectomy and cycloheximide treatment on subsequent growth patterns of DMBA-induced mammary tumours

Administration of cycloheximide within 10 min of ovariectomy did not prevent the histologically classified [7] "regression process" observed at day 7, postovariectomy. Cycloheximide did however prevent to some degree, the reduction in tumour mass measured 3 weeks later (Fig. 4). No altered tumour growth patterns were seen following the administration of cycloheximide to intact animals.

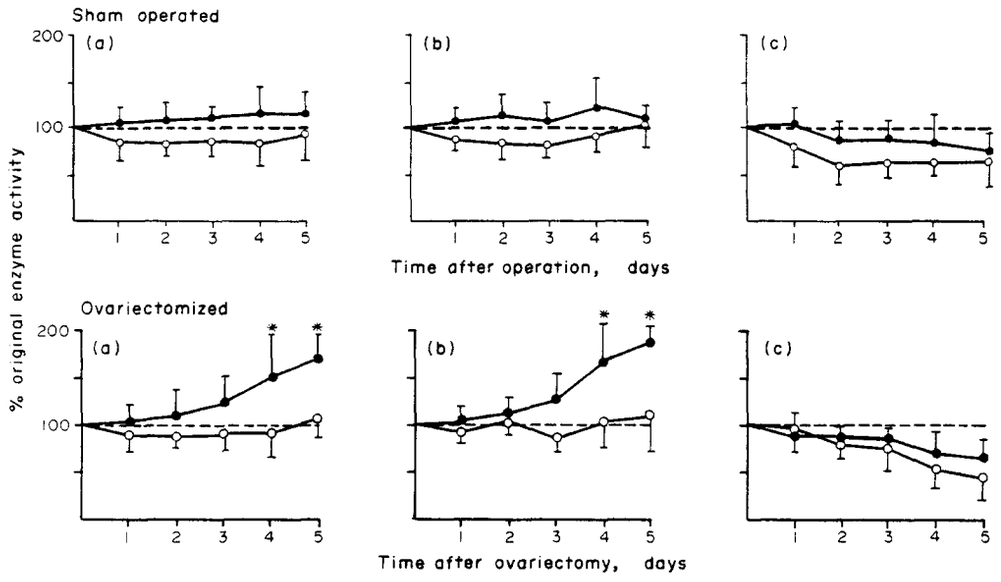

Fig. 3. Effect of ovariectomy and cycloheximide treatment on enzyme activities present within mammary tumours. Groups of animals were ovariectomized and given a single i.m. injection of cycloheximide (0.5 mg/kg *body weight*) *within* 10 min *of the operation. Control animals were sham operated. Tumour tissue was biopsied at the time of the operation (prior to cycloheximide treatment) and on* 5 *subsequent days. Approximately* 100 mg *of tissue was removed with each biopsy and portions homogenized and assayed for (a) p-nitrophenylphosphatase, (b) β-glucuronidase and (c) glucose-6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase. The results are the mean ± S.D. of* 5 *tumours per group and activity is expressed as a percentage of the original activity (C), (●——●, ovariectomized and ○——○, ovariectomized and treated with cycloheximide, *pVc<0.05 (P values were estimated by Students "t"-test for independent means).*

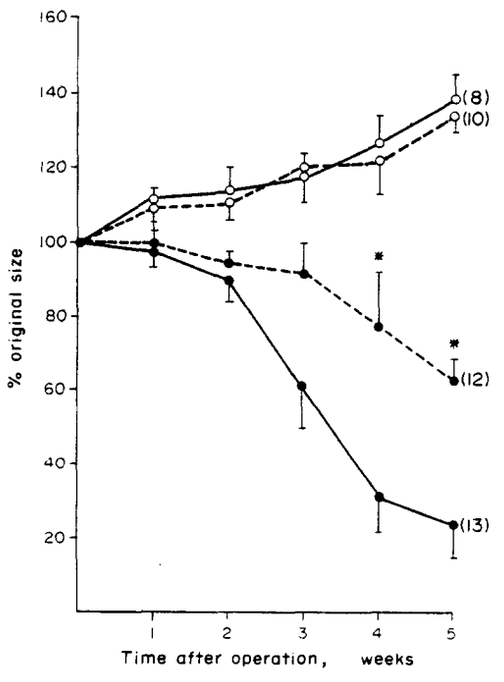

Fig. 4. Effect of ovariectomy and cycloheximide treatment on subsequent growth patterns of mammary tumours. Groups of animals were ovariectomized and given a single i.m. injection of cycloheximide (0.5 mg/kg *body weight*) *within* 10 min *of the operation. Control animals were sham operated. Tumour size was measured weekly as the mean of two diameters. Control,* 0, *ovariectomized* (O), ●, *cycloheximide treated,* -----. **pVO<0.05 (P values were determined using a Students "t"-test for independent means). Figures given in parenthesis indicate number of tumours per group.*

DISCUSSION

One of the most important features of the DMBA-induced rat mammary carcinoma is that the majority of these tumours regress after either ovariectomy [13] or hypophysectomy [14]. The results now described clearly show that after ovariectomy the activities of the various enzymes studied in "regressing" DMBA-induced mammary tumours were found to be different from values determined in "growing" or autonomous tumours. Such enzyme assays, therefore, offer a means by which tumour regression could be monitored and reflect also the hormone dependency of the tissue. The histologically recognized "regression" in ovariectomized animals was characterized by increased specific activities for the enzymes *p*-nitrophenylphosphatase and β-glucuronidase. These enzymes showed elevated activities 5 days after ovariectomy in spite of reduced levels of protein synthesis occurring within the tumour tissue at this time. The time sequence between the increase in lysosomal enzyme activity and the regression of tumour mass would suggest that these enzymes were concerned in the process. This was further emphasized by studies with cycloheximide, an inhibitor of protein synthesis [15–17]. Administration of the inhibitor to ovariectomized animals within 10 min of the

operation, decreased [^{3}H]-leucine incorporation *in vitro* into tumour protein, inhibited the elevated activities of the lysosomal enzymes studied and reduced the decrease in tumour mass resulting from the operation. These results indicate that the processes which govern the elevation of acid hydrolase synthesis are initiated on day 1 postovariectomy and that the levels obtained relate to the regression produced. Since changes in lysosomal enzyme activity were not observed until approximately 3 days post-ovariectomy, it is possible that the triggering events are not initially directed towards acid hydrolase synthesis, rather they operate through an intermediary process.

Weekly palpatation of the hormone-dependent tumours showed that no significant decrease in tumour size occurred until the third week after ovariectomy, at which time tumour size had decreased 40%. The result is in agreement with Hilf *et al.* [18]. Recent studies by Gullino *et al.* [19–22] have failed to show any marked lysosomal enzyme activity alterations prior to changes in tumour size. They have also been unable to affect tumour regression by administration of actinomycin D, cycloheximide and puromycin [22]. In those studies, however, physical regression, to about 50% the original size, was observed after 72 hr. Clearly the results indicate considerable variations in the rates and the responses of tumour regressions to those described here. The differences may reflect the hormone environment of tumours in animals from different laboratories. Such a difference with respect to plasma prolactin concentrations has been postulated to effect the incidence of DMBA-induced mammary tumours in varying strains of rats [23].

It seems likely, from the present data, that the elevation of acid hydrolase activity involves at least some *de novo* synthesis of enzyme protein and is not totally mediated through alterations in the chemical properties of the enzymes [9]. The evidence of Lanserotti and Gullino [21] supports these results. They demonstrated that the increased activity of the lysosomal enzymes RNAase, β-glucuronidase, β-galactosidase and acid phosphatase seen during the regression of the transplantable MTW9 mammary tumour and also the DMBA-induced mammary tumour coincided with an increased quantity of enzyme as measured by a radial diffusion immunoelectrophoresis technique.

Tumour regression following ovariectomy was also characterized by an apparent decrease in the activity of enzymes related to carbohydrate metabolism. Reduced activity of the enzymes glucose-6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase was demonstrated by day 5 postovariectomy. Unlike the lysosomal acid hydrolase response to ovariectomy where activities determined in "regressing" tissue approached normal levels after approximately 3 weeks, the pyridine nucleotide-linked dehydrogenase levels continued to decrease over the experimental period. Similar results have been observed in the DMBA-induced mammary tumour by Hilf *et al.* [18] and in the 3-methylcholanthene-induced mammary tumour by Rees and Huggins [24] following ovariectomy. Administration of cycloheximide did not prevent the decrease of the pyridine nucleotide-linked dehydrogenases caused by ovariectomy. The results may indicate therefore that lysosomal enzymes are not directly involved in the process by which the activity of these enzymes are reduced, although cathepsin B_1, an enzyme of probable lysosomal origin in the DMBA-induced tumour [25], has been shown to inactivate certain pyridine nucleotide-linked dehydrogenases [26].

Further studies are now in progress to examine more closely the role of these enzymes in the regression process of mammary tumours. It is hoped that a greater understanding of the molecular events leading to alterations in tumour mass will be useful in therapeutic approaches to human breast cancer.

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A Method for the Quantitative Detection of Human Acute Lymphatic Leukemia

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Abstract—*Lymphocytes isolated from the peripheral blood of* 32 *patients with acute lymphatic leukemia (ALL),* 4 *patients with non-leukemia malignant disorders and from* 12 *normal healthy donors were studied for fluidity of membrane lipids. The degree of fluidity of cellular membranes was determined by fluorescence polarization analysis using the fluorescent probe* 1,6-*diphenyl* 1,3,5-*hexatriene (DPH), when embedded in membrane lipid region of intact cells. Based on the fluorescence polarization* (P) *analysis the degree of membrane microviscosity* ($\bar{\eta}$) *was determined. Results have shown that lymphocytes isolated from untreated ALL patients have a more fluid lipid layer in their cellular membrane (average of* 17 *patients* $\bar{\eta} = 2.65$ *poise at* 25°C) *than lymphocytes isolated from normal donors (average of* 12 *donors* $\bar{\eta} = 3.91$ *poise at* 25°C). *The results also showed that the degree of membrane lipid microviscosity of lymphocytes isolated from ALL patients in partial or complete clinical remission is similar to that of normal lymphocyte (average of* 15 *patients* $\bar{\eta} = 3.89$ *poise at* 25°C). *Lymphocytes isolated from patients with non-leukemic malignant disorders showed a similar characteristic to normal lymphocytes. These results which confirmed our previous observations with other leukemias are in line with the suggestion that fluorescence polarization analysis of DPH labelled lymphocytes can serve as a quantitative clinical tool for human acute lymphatic leukemia.*

INTRODUCTION

IT SEEMS to be generally agreed that analysis of differences in the dynamic structural organization [1, 2] of membrane components in normal and malignant cells is an important tool in the study of leukemogenesis and carcinogenesis [3–5]. Among the membrane changes that are associated with malignant transformation of normal cells, dynamic characteristics, such as the degree of mobility of membrane protein receptors [6] or the degree of fluidity of membrane lipids [7], can be quantitatively evaluated by fluorescence polarization analysis [8]. Study of these changes, therefore, can be of value for the development of quantitative methods for distinguishing between normal and malignant cells.

Previous studies have led to the development of a quantitative techniqie for the determination of the degree of fluidity of membrane lipids in normal and leukemic lymphocytes [7, 9, 10] and in normal and transformed fibroblasts [11–13], both on a population level and on a single cell level [14]. This method is based on fluorescence polarization analysis of the fluorescent probe, 1,6-diphenyl 1,3,5-hexatriene (DPH) when embedded in the hydrophobic core of membrane lipids of intact cells. With the aid of this method it has been shown that the development of leukemia both in experimental animals and humans is accompanied by a marked increase in the degree of fluidity of membrane lipids in the leukemic cells as compared to normal lymphocytes [15, 16]. The difference in the degree of lipid fluidity between normal and leukemic lymphocytes is determined to a large extent by a difference in the lipid composition of their cellular membranes [17].

The main issue of our present experiments was to determine the degree of membrane lipid fluidity of human normal and leukemic lymphocytes in order to establish a reliable and quantitative tool for the detection and prognosis of human acute lymphatic leukemia.

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MATERIAL AND METHODS

Patients. Blood samples for these studies were obtained from hospitalized patients with acute lymphatic leukemia (ALL) at the IGR and at I.C.I.G. Villejuif, France. Samples of peripheral blood were obtained from ALL patients, patients with non-leukemic malignant disorders and from normal donors, including treated and untreated cases.

Isolation of cells. Nucleated cells were isolated from the blood samples by the Ficoll–Hypaque gradient centrifugation. For isolation a Ficoll–Hypaque solution was prepared by mixing 24 ml of a 9% Ficoll-400 (Pharmacia, Sweden) solution in distilled water with 6.8 ml Hypaque sodium 50%, brand sodium diatrizoate (Winthrop Co., U.S.A.) with 3.2 ml distilled water. Fresh blood was diluted 1:1 or 1:2 with phosphate-buffered saline (PBS) (pH 7.2) and a volume of 10 ml diluted blood was layered on 3 ml of the Ficoll–Hypaque solution. The samples were centrifuged for 20 min at 500 x *g* at 25°C and the isolated lymphocytes were washed twice with PBS and diluted with PBS to the appropriate concentration. Blood samples and isolated cells were kept at 4°C.

Labelling of cells with DPH. The fluorescent hydrocarbon 1,6-diphenyl 1,3,5-hexatriene (DPH) was used in the present study as a fluorescence probe for monitoring the degree of fluidity in the cellular membrane lipids. Labelling of cells was performed with a 2×10^{-6} M DPH dispersion in PBS obtained by injection of 0.1 ml of 2×10^{-3} M DPH in tetrahydrofuran into 100 ml PBS which has been vigorously stirred. This DPH dispersion is practically clear and void of fluorescence [7]. A volume of 2 ml cell suspension in PBS at a concentration of 4×10^6 cells/ml was incubated with 2 ml DPH dispersion for 60 min at 25°C. The labelled cells were then washed twice with PBS, resuspended in PBS at a concentration of 2×10^6 cells/ml and immediately used for the fluorescence studies. Similar results were obtained when cells were labelled with a DPH dispersion where the 0.1 ml tetrahydrofuran was evaporated by nitrogen.

Fluorescence polarization analysis. The degree of lipid fluidity was quantitatively determined by fluorescence polarization, *P*, analysis of the DPH labelled cells. Experiments were carried out with the Elscint Microviscosimeter, Model MV-1 (Elscint Ltd., Haifa, Israel). Excitation was performed with a polarized 365 nm band generated from a 200 W mercury arc, and the emitted light was detected in two independent cross-polarized channels equipped with cut-off filters for wavelengths below 390 nm. The degree of fluorescence polarization, *P*, and the fluorescence anisotropy, *r*, were obtained according to the following equations:

$$P = \frac{I_v - I_h}{I_v + I_h} \qquad \text{(a)}$$

$$r = \frac{I_v - I_h}{I_v + 2I_h}, \qquad \text{(b)}$$

where I_v and I_h are the emission intensities polarized vertical and horizontal to the direction of polarization of the exciting light, respectively. High *P* values represent low lipid fluidity, whereas low *P* values represent high lipid fluidity [7]. All fluorescence measurements with DPH in the present study were carried out at 25°C. The accuracy of the *P* values obtained with the microviscosimeter was $P = \pm 0.005$. The method employed in the present study for the evaluation of membrane microviscosities is based on the fluorescence polarization properties of a fluorescence probe as described by the Perrin equation:

$$\frac{r_0}{r} = 1 + C(r)\frac{T \cdot \tau}{\bar{\eta}}, \qquad \text{(c)}$$

where *r* and r_0 are the measured and the limiting fluorescence anisotropies, T is the absolute temperature, τ is the excited state lifetime of DPH and $\bar{\eta}$ is the microviscosity of the medium where the DPH molecules are embedded [7]. High *P* values correspond to high $\bar{\eta}$ values and indicate a low lipid fluidity and vice versa. The fluorescence lifetime, τ, of DPH in human normal and leukemic lymphocytes at 25°C was 10.0 ± 0.7 nsec as determined with the aid of an Ortec photon-counting fluorescence instrument. These values are in good agreement with those described for mouse normal and leukemic lymphocytes [17].

RESULTS

The degree of fluidity of membrane lipids of cells isolated from human peripheral blood was quantitatively determined by monitoring the degree of fluorescence polarization, *P*, of DPH embedded in lipid regions of intact cells. Based on the *P* values which were obtained by a direct measurement the degree of membrane micro-

viscosity, $\bar{\eta}$ was calculated. High P or $\bar{\eta}$ values indicate a low lipid fluidity and vice versa. The present studies were carried out with cells isolated from untreated and treated acute lymphatic leukemic (ALL) patients, patients with non-leukemic malignant disorders, and normal healthy donors. The results indicate (Table 1 and Fig. 1) that blood cells isolated from untreated ALL patients or ALL patients in relapse exhibit low P values: range $P=0.224$–0.265; average $P=0.250$ (Table 1). These low P values correspond to low microviscosity, $\bar{\eta}$, values (high lipid fluidity): range $\bar{\eta}=2.00$–3.05 poise; average $\bar{\eta}=2.65$ poise. The low P and $\bar{\eta}$ values of cells isolated from untreated ALL patients increases in lymphocytes isolated from ALL patients undergoing clinical remission (Table 2 and Fig. 1). The P values of cells obtained from ALL patients in remission (no blasts in the peripheral blood and less than 5% blasts in the bone marrow) were: range $P=0.275$–0.309; average $P=0.288$ (Table 2) which correspond to the following $\bar{\eta}$ values: range $\bar{\eta}=3.37$–4.80 poise; average $\bar{\eta}=3.89$ poise (Fig. 1). These results have indicated that the mean degree of membrane lipid fluidity decreases in blood cells isolated from ALL patients undergoing clinical remission as compared to cells obtained from untreated ALL patients and from ALL patients in relapse. It is of great interest to point out that blood cells isolated from ALL patients in partial remission (more than 5% blasts in the bone marrow) exhibit membrane fluidity values in between those which are characteristic for cells isolated from untreated ALL patients and from ALL patients in complete remission (Table 1 and Table 2).

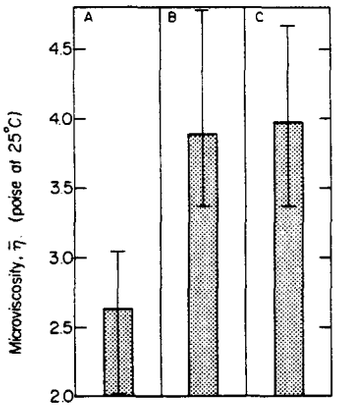

Fig. 1. The range and the average membrane microviscosity of lymphocytes isolated from the peripheral blood of: A—untreated ALL patients (Table 1); B—ALL patients in remission (Table 2); C—normal donors (Table 3) and patients with non-leukemic malignant disorders (Table 4).

Lymphocytes isolated from normal donors (Table 3) or from patients with non-leukemic malignant disorders (Table 4) exhibit P and $\bar{\eta}$ values similar to those obtained with cells isolated from ALL patients who undergo complete clinical remission. The average membrane microviscosity, $\bar{\eta}$, values were 2.65; 3.89 and 3.91 poise at 25°C for untreated ALL patients (17 patients); ALL patients in clinical remission (15 patients); and normal donors (12 donors), respectively (Fig. 1). It is of importance to notice that the upper limit of $\bar{\eta}$ values in blood cells isolated from untreated ALL patients is below the lower limit of the $\bar{\eta}$ value of lymphocytes

Table 1. The measured degree of fluorescence polarization, P, and the calculated degree of membrane microviscosity, $\bar{\eta}$, of lymphocytes isolated from peripheral blood of 17 untreated ALL patients

Patient	Degree of fluorescence polarization, P, at 25°C	Membrane microviscosity, $\bar{\eta}$, poise at 25°C
PR	0.243	2.44
AN	0.256	2.79
CA (F)	0.260	2.90
DA (F)	0.248	2.56
CO	0.259	2.87
EL	0.259	2.87
GO	0.265	3.05
PO	0.265	3.05
HI (F)	0.246	2.52
NE (F)	0.235	2.26
TA	0.224	2.02
GA	0.236	2.28
DU	0.241	2.40
PI	0.250	2.63
KR	0.255	2.76
LE	0.259	2.87
BE	0.259	2.87

F = Frozen cells.

Table 2. The measured degree of fluorescence polarization, P, *and the calculated degree of membrane microviscosity,* $\bar{\eta}$, *of lymphocytes isolated from peripheral blood of* 15 *ALL patients in remission*

Patient	Degree of fluorescence polarization, *P*, at 25°C	Membrane microviscosity, $\bar{\eta}$, poise at 25°C
GI (CR)	0.287	3.83
DE (CR)	0.294	4.10
HU (CR)	0.289	3.89
CA (PR)	0.280	3.55
CA (CR)	0.299	4.30
BA (PR)	0.283	3.64
SP (PR)	0.284	3.67
SP (CR)	0.297	4.24
DI (F) (CR)	0.290	3.93
FR (PR)	0.291	3.59
PR (PR)	0.282	3.63
LA (F) (PR)	0.275	3.37
HA (F) (CR)	0.309	4.80
RO (CR)	0.292	4.01
GA (CR)	0.290	3.93

PR = Partial remission.
CR = Complete remission.
F = Frozen cells.

Table 3. The measured degree of fluorescence polarization, P, *and the calculated degree of membrane microviscosity,* $\bar{\eta}$, *of lymphocytes isolated from peripheral blood of* 12 *normal donors*

Donor number	Degree of fluorescence polarization, *P*, at 25°C	Membrane microviscosity, $\bar{\eta}$, poise at 25°C
1	0.291	3.97
2	0.288	3.85
3	0.291	3.97
4	0.292	4.01
5	0.292	4.01
6	0.300	4.35
7	0.279	3.51
8	0.300	4.35
9	0.275	3.37
10	0.287	3.83
11	0.290	3.93
12	0.287	3.83

Table 4. The measured degree of fluorescence polarization, P, *and the calculated degree of membrane microviscosity,* $\bar{\eta}$, *of lymphocytes isolated from peripheral blood of* 4 *patients with non-leukemic malignant disorders*

Patient	Disorder.	Degree of fluorescence polarization, *P*, at 25°C	Membrane microviscosity, $\bar{\eta}$, poise at 25°C
BL	Breast cancer	0.290	3.93
AR	Ovary Cancer	0.307	4.69
BI	Hepatoma	0.289	3.89
TU	Breast Cancer	0.288	3.85

obtained from ALL patients in remission, normal donors and patients with non-leukemic malignant diseases (Fig. 1).

Some of the patients in the present study were followed for periods of up to 4 weeks and their cells examined on several occasions using DPH. The results summarized in Fig. 2 indicate that the degree of fluorescence polarization of DPH

obtained from different samples of blood cells from the same patient was generally in good agreement and corresponded well with the steady state of the disease.

DISCUSSION

In the present study we have employed fluorescence polarization analysis in order to establish a quantitative method for the prognosis of human acute lymphatic leukemia (ALL). The results of our studies clearly indicate that cells isolated from peripheral blood of untreated ALL patients or from ALL patients in relapse can be characterized by an increase in the degree of fluidity of the membrane lipids (decrease in membrane microviscosity) as compared to cells isolated from ALL patients undergoing clinical remission or from normal donors. These results are in line with previous studies where it has been shown that the increase in membrane lipid fluidity of leukemic cells both from experimental animals and humans is apparently mainly due to a decrease in the mole ratio of cholesterol to phospholipids in membranes of the leukemic cells as compared to normal lymphocytes [7, 9, 10, 14, 16, 17]. Further studies will include a detailed cytological analysis of the blood cells.

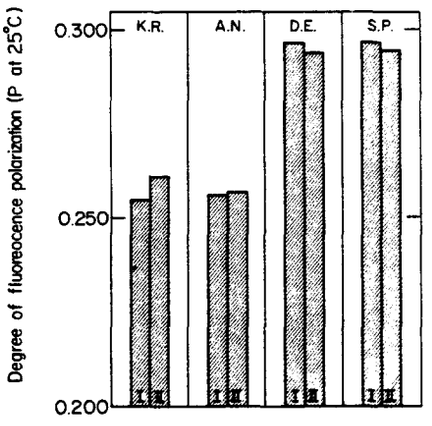

Fig. 2. Degree of fluorescence polarization, P, *of DPH labelled lymphocytes isolated at different periods (I,II) from the same patient.*

The results of previous and present studies in human leukemia indicate that one of the basic parameters that changes in leukemic cells as compared to normal lymphocytes is the lipid composition of cellular membranes, mainly the cell surface membrane [15, 17]. These changes in the lipid composition of the cell membrane determine to a large extent its dynamic behavior. The major dynamic change induced by changes in the lipid composition of the cellular lipid layers is on the fluidity/rigidity nature of the surface membrane lipid core [17]. One of the most sensitive methods now available to determine the degree of membrane lipid fluidity in mammalian cells is fluorescence polarization analysis of DPH labelled intact cells [7]. By using this method, the fluidity/rigidity behavior of cellular membrane lipids can be quantitatively monitored [7, 10, 11, 13, 14]. Based on our present results, which confirmed previous observations, it is suggested that fluorescence polarization analysis of DPH labelled normal and leukemic cells can serve as a quantitative clinical tool for the prognosis of human acute lymphatic leukemia.

Moreover, studies attempting to elucidate the biological and clinical significance of membrane lipid fluidity and of the cholesterol to phospholipids (C/PL) ratio in cellular membranes showed that the reduction of membrane fluidity of mouse leukemic cells induced by an *in vitro* introduction of exogenous cholesterol into the surface membrane resulted in a marked inhibition of their tumorigenicity *in vivo* [18, 19]. It has also been suggested that in human leukemia cholesterol, but not phospholipids, in cellular membranes is exchangeable with cholesterol in the serum lipoproteins and therefore concomitant to the cellular reduction in C/PL, the C/PL average in the blood serum of leukemic patients is below the average normal C/PL ratio [15]. Experimental evidence to support this hypothesis have been recently obtained by *in vitro* incubation of human leukemic cells in normal serum [16]. The results of these experiments have shown that there is a rapid translocation of cholesterol from the normal serum to the leukemic cells that changes the membrane fluidity from the leukemic behavior to that characteristic for normal lymphocytes [16]. Based on these observations, it is now suggested to test the possibility of whether or not increase in the C/PL molar ratio both in the blood serum of leukemic patients and in the leukemic cell membranes up to the normal C/PL ratio will result in a clinical remission. The interrelationships between membrane lipid fluidity, C/PL ratio in the cell membrane and in the blood serum and the normal and malignant behavior of human normal lymphocytes and leukemic cells is as yet hypothetical in many respects and requires further investigation. Research along some of these lines is presently being carried out by our groups.

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Childhood Malignant Lymphoma. Favourable Outlook with Aggressive Combination Chemotherapy and Radiotherapy

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Abstract—*From* 1963–1974, 62 *cases of malignant lymphoma were treated:* 24 *patients with Hodgkin's lymphoma (HL) and* 38 *with non-Hodgkin's lymphoma (NHL). Relapse rate in children with HD before* 1971 *(pre-laparotomy data) was significantly higher than in the group after* 1971, *when explorative laparotomy, splenectomy and more aggressive radio- and chemotherapy were applied* (10/15 *vs* 2/9). *Since* 1970 *all children with NHL were entered in a modified leukaemia protocol combining radiotherapy to the primary site, prophylactic C.N.S.-irradiation and aggressive multiple drug chemotherapy. In this group* 23/26 *patients achieved complete remission vs* 7/12 *before* 1970, *when therapy consisted of low dose irradiation to the primary site, and/or single agent chemotherapy. Median survival in the group before* 1970 *was* 5 *months compared with* 34+ *months after* 1970. *Only* 1 *patient receiving prophylactic C.N.S.-irradiation developed C.N.S.-leukaemia vs* 5/12 *in the group before* 1970.

INTRODUCTION

WITH the introduction of explorative laparotomy and splenectomy in 1969 the diagnostic approach to Hodgkin's disease changed considerably [1]. More aggressive radiotherapy in the early stages and the use of multiple drug combinations since the late 1960's have revolutionized treatment programmes [2, 3]. Whereas Hodgkin's lymphoma (HL) with its distinct histological features has a similar biological behaviour in adults and children, non-Hodgkin's lymphoma (NHL) in childhood differs from lymphomas in adults in its pattern of spread, following the course of acute lymphoblastic leukaemia. This provided the rationale for treatment programmes successful in the latter disease.

This retrospective analysis was undertaken to compare the results of different treatment programmes in children with malignant lymphoma.

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MATERIAL AND METHODS

Between 1 January, 1963 and 31 December, 1974, 62 patients with malignant lymphoma were diagnosed at Children's Hospital, University of Munich: 24 had HL and 38 had NHL.

Hodgkin's disease

Among the 24 patients 18 were male and 6 female. The youngest patient was 2 yr 6 months, the oldest 15 yr. Half of the patients were under 10 yr. Histological classification yielded lymphocytic predominance (3), nodular sclerosis (4), mixed cellularity (12), lymphocytic depletion (1), and lymphogranulomatosis without any further specification (4).

Non-Hodgkin's lymphoma

Among the 38 patients, 28 were male and nearly half of the children were under 10 years. Initially our lymphomas were classified as lymphosarcomas and reticulum cell sarcomas. Retrospective analysis using the "Kiel Classification" [4, 5] showed, that all lymphomata were of high grade malignancy. In the group before 1970 6 children had lymphoblastic and one immunoblastic lymphoma. In 5 patients a

retrospective histologic classification was not possible. In the group after 1970 18 children had lymphoblastic and 4 immunoblastic lymphoma, in 4 patients no retrospective classification could be carried out.

Clinical staging was done retrospectively, according to the staging system by Wollner *et al.* [6], which is a modification of the Ann Arbor classification [7]. None of our patients had lymphangiograms, staging laparotomy or radioactive isotope scans. Bone marrow aspirates, however, no marrow biopsies, were performed in all children. In case of bone marrow involvement, criteria for diagnosis of non-Hodgkin's lymphoma versus acute lymphoblastic leukaemia was the demonstration of either a bulky primary tumor in a nodal or an extranodal site. Since 1970 spinal taps were performed regularly at the time of diagnosis.

The distribution by stage was as follows: Stage I (8), stage II (6), stage III (10), stage IV (14).

TREATMENT

Hodgkin's disease

In 1971 explorative laparotomy and splenectomy were incorporated in the diagnostic and therapeutic programme. Before 1971 the diagnosis of abdominal involvement could only rely on physical findings and lymphangiograms. Since then the extent of disease was defined more precisely by splenectomy, liver and lymphnode biopsies. In addition radiation was changed from involved field therapy to extended field therapy, the extent depending on the pathological stage. Also single agent chemotherapy was abandoned in favour for combination chemotherapy with cyclophosphamide, vinblastine, procarbazine and prednisone.

Non-Hodgkin's lymphoma

Between January 1964 to December 1969 all patients were treated with low dose irradiation to the primary and single agent chemotherapy in most cases. No consequent maintenance therapy followed remission. Since January 1970 all children with non-Hodgkin's lymphoma, regardless of stage and histology, were entered in a modified leukaemia protocol, using intensive induction therapy with vincristine, prednisone and cyclophosphamide, concurrent with megavoltage therapy to the primary with 3000–4000 rad Co^{60}. As soon as clinical remission was obtained, C.N.S.-irradiation with 2400 rad to the cranium and intrathecal methotrexate were given in most cases. Simultaneously oral therapy with 6-mercaptopurine daily and cyclophosphamide weekly was started. After C.N.S.-prophylaxis, methotrexate p.o. was added. According to protocol $2\frac{1}{2}$ yr of chemotherapy are given.

RESULTS

Hodgkin's disease

Out of 15 children before 1971, 11 achieved complete and four partial remission. In 10 patients the disease recurred. Five children of the total group are still living in their first remission, off therapy (median 78+ months). In the group of 9 patients since 1971, 8 entered complete remission. Two children relapsed. No patient had complications which could be solely attributed to splenectomy. Also, we did not encounter a higher incidence of infections or of severe complications with aggressive therapy than in the first group without aggressive therapy.

Non-Hodgkin's lymphoma

Seven of twelve patients before 1970 achieved complete remission. Five children developed C.N.S.-leukaemia. Median survival for all patients was 5 months. No patient survived (Table 1). In the group since 1970, 23/26 patients showed complete remission. Thirteen patients (50%) are alive with no evidence of disease with a median survival of 34+ months. Eight of these children are still in their first complete remission and 5 in their second complete remission. Four children are off therapy from 22 to 48 months (Table 2). Only one patient with C.N.S.-prophylaxis developed C.N.S.-leukaemia. All patients with the primary site in the mediastinum relapsed, but one.

Table 1. Non-Hodgkin's lymphoma (NHL)—patients before 1970

Stage	Patients achieving complete remission	Patients surviving
I	2/2	0/2
II	0/0	0/0
III	2/3	0/3
IV	3/7	0/7
All patients	7/12	0/12 Median survival 5 months

DISCUSSION

In agreement with data of other centres, our patients with Hodgkin's disease were pre-

Table 2. Non-Hodgkin's lymphoma (NHL)—patients 1970

Stage	Patients achieving complete remission	Patients surviving (NED) median survival (range) in months	Patients in first remission median (range) in months
I	6/6	6/6 50.5^+ ($16^+ - 63^+$)	5/6 46^+ ($16 - 61^+$)
II	5/6	3/6 13.5^+ ($3 - 51^+$)	2/6 42.5^+ ($34^+ - 51^+$)
III	6/7	2/7* 6.5^+ ($2 - 26^+$)	1/7 26^+
IV	6/7	2/7* 7.5^+ ($0.5 - 39^+$)	0/7 46^+ ($16 - 61^+$)
All patients	23/26	13/26 34^+ ($16 - 63^+$)	8/26

NED = no evidence of disease.
* = one lost to follow up.

dominantly male. Also mixed cellularity was the most frequent histological subgroup. Though Hodgkin's disease is rare below the age of five, it should not be omitted from differential diagnosis in this age group. At time of diagnosis 3 of our 24 patients were $2\frac{1}{2}$, 4 and $4\frac{1}{2}$ yr respectively.

Poor results in the group of patients before 1971 with only one third remaining in their first remission, led to a more aggressive approach. Staging laparotomy and splenectomy in our experience were not followed by more serious side-effects. However, it seems to be difficult to evaluate accurately whether infectious complications after splenectomy in Hodgkin's disease are due to the missing spleen, chemotherapy or to immunologic alterations inherent to the disease [8].

Though the disease is potentially curable with radiotherapy in the early stages, patients of stage I and II with unfavourable localization, histology, and systemic symptoms have a high risk of extranodal relapse ranging between 20–60% [9, 10]. Apart from these considerations rationale for chemotherapy even in early stages was based on the possibility, that microfoci below the diaphragm may be overlooked in spite of laparotomy. With this approach, the relapse rate of 10/15 patients before 1971 could be reduced to 2/9 patients after 1971. However, because of the small number of patients and the short period of observation, definite conclusions cannot be drawn.

Non-Hodgkin's lymphoma in childhood is known for its propensity to involve bone marrow and to spread to the meninges, a biological behaviour known with acute lymphoblastic leukaemia [11, 12]. Half of the patients can be expected to manifest leukaemic transformation within the first year followed by C.N.S.-disease. Thus survival rates in non-Hodgkin's lymphoma in childhood reported by several investigators in the 1960's were very discouraging [12–14].

In agreement with recent reports [15, 16] most of our patients in both groups had lymphoblastic lymphoma (24/29), which according to Lennert and the "Kiel classification" of malignant lymphomas includes acute lymphoblastic leukaemia (ALL) [4]. The small number of cases with immunoblastic lymphoma does not allow conclusions regarding the influence of histology on prognosis.

At present the best results in ALL are achieved with vincristine and prednisone induction, maintenance combination chemotherapy, and C.N.S.-prophylaxis mostly in the form of cranial irradiation and intrathecal methotrexate [17]. It consequently seems obvious to employ these principles for the treatment of all NHL in children [15, 18]. Our present results justify this aggressive approach: 88% of the patients treated in this manner achieved complete remission compared with 50% before 1970. Whereas all patients with a less aggressive treatment died, 50% of our patients in the second group are alive with no evidence of disease. These data compare favourably with results of other centres employing combined treatment programmes in childhood non-Hodgkin's lymphoma.

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Total Body Irradiation in Advanced Non-Hodgkin's Lymphoma

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Abstract—*Twenty-four patients with Stage* III *and* IV *non-Hodgkin's lymphoma were treated by total body irradiation (TBI). Complete clinical and radiological regression of disease (CR) was achieved in* 13 *patients* (54.2%). *The duration of complete remission ranged from* 3.5 *to* 26 *months (mean* 9.7 *months), including five patients disease-free at the time of reporting. On the basis of these preliminary observations a regime employing repeated courses of TBI is being evaluated.*

INTRODUCTION

RECOGNITION of the widespread nature of many forms of human malignant disease led naturally to the concept of TBI early in the history of radiation therapy. Dessauer [1] for example employed this approach within 12 yr of the discovery of X-rays. Heublein [2] designed a special unit for the purpose in which a 135 kV X-ray machine was in continuous operation. Medinger and Craver [3] treated more than 200 patients with a variety of tumours and reported responses in lymphoma patients. Systemic radiotherapy was explored and utilized in the 1940's and 1950's by Osgood [4] for the treatment of the chronic leukaemias. More recently the study of low dose rate TBI has been in progress at Oak Ridge [5]. Renewed interest in TBI for malignant lymphomas is due to the recent published work of Johnson [6–9] who has reported that small fractions of megavoltage radiation to the whole body may induce complete remissions in both lymphocytic lymphoma and chronic lymphocytic leukaemia.

This paper reports our experience with TBI in 24 patients with Stage III and IV non-Hodgkin's lymphoma.

MATERIAL AND METHODS

1. *Histological classification*

The Rappaport classification (1966) [10] was employed.

Lymphocytic lymphoma. Well-differentiated nodular—WDLN. Well differentiated diffuse—WDLD.

Lymphocytic lymphoma. Poorly differentiated nodular—PDLN; Poorly differentiated diffuse—PDLD. Mixed lymphocytic/histiocytic, nodular—NMX, diffuse—DMX. Histiocytic nodular—NH; Histiocytic diffuse—DH; Diffuse undifferentiated—DU.

2.

Staging was according to the Ann Arbor system [11].

3. *Patients*

Twenty-four patients were treated. Details of sex, age, stage and his ology are shown in Tables 1–3.

There were 11 Stage III patients and 13 Stage IV. Sixteen patients had lymphocytic lymphoma (seven nodular, seven diffuse), three patients had diffuse undifferentiated lymphoma and five nodular mixed lymphoma. Three of the Stage IV patients had extensive skin lymphoma.

4. *Patient investigation*

This included lymphography, i.v. urography, chest radiograph, marrow aspirate and trephine biopsy and liver function tests.

5. *Criterion for therapy*

Following patient assessment no therapy was instituted in cases of nodular lymphoma until there was definite evidence of tumour progression (usually nodal enlargement on the abdominal film).

6. *Treatment protocol*

Radiation therapy was covered by Allopurinol (300 mg/day) to prevent uric acid nephropathy. Three irradiation regimes were employed and these are summarized in Table 4.

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Table 1. Hemibody irradiation Cr 4/8 (Range 4.5–26 months)

Patient	Age	Stage	Histology	Previous treatment	Response to HBI
1	48	IV B m+	NMx	CT & RT	CR, 4½ months
2	61	III A	PDLN	—	CR, 16 months
3	56	IV m+	NMx	RT	CR, 17 months*
4	51	III A	PDLN/D	RT	—
5	38	III A	NMx	—	—
6	41	III A	PDLN	RT	CR, 26 months
7	35	IV B	MDLD	CT	—
8	58	IV m+	PDLD	CT & RT	—

*Remaining in complete remission.

Table 2. Protracted total body irradiation Cr 5/7 (Range 3–13 months)

Patient	Age	Stage	Histology	Previous treatment	Response to PTBI
9	60	III A	MDLN	—	CR, 13 months
10	69	III E	MDLD	—	—
11	68	IV A	DU	CT	—
12	54	IV m+	MDLD	—	CR, 6 months†
13	55	IV A	WDLD	—	CR, 3 months†
14	68	IV A	WDLD	—	CR, 4 months†
15	55	IV A	WDLD	—	CR, 3 months†

†Remaining in complete remission.

Table 3. Total body irradiation Cr 4/9 (Range 6.5–10.5 months)

Patient	Age	Stage	Histology	Previous treatment	Response to TBI
16	54	III A	DU	CT & RT	—
17	67	IV A	MDLN	—	CR, 6.5 months
18	67	IV A	DU	—	—
19	43	III A	MDLN	—	18
20	55	III A	NMx	—	CR, 9.5 months
21	57	III A	NMx	—	CR, 10.5 months
22	35	IV m+	WDLN	—	CR, 7.0 months‡
23	56	III B	PDLN	—	—
24	72	IV A	MDLD	CT & RT	—

‡Remaining in complete remission.

Table 4. Total body irradiation protocols

1. *Total Body Irradiation* (TBI)
   Mid plan dose 150 rad given in 10 fractions over 12 days.

2. *Protracted TBI* (PTBI)
   Mid plan dose 150 rad given in 10 fractions twice weekly over 5 weeks.

3. *Hemibody Irradiation* (HBI)
   300 rad mid plan dose given to one half of the body in 10 fractions over 12 days.
   6–8 weeks later the remaining half is treated in the same way.

Patients were irradiated in the sitting position with arms crossed in front of the chest and knees flexed with tissue equivalent rubber between the legs to approximate leg thickness to that of the body. No part of the body was shielded. Because of the difference of the head and neck thickness to that of the body, the mid-plane cranial dose was 15–25% higher than that of the mid-pelvis. Doses were checked in all cases using lithium fluoride thermoluminescent dosimetry. Both sides of the body were treated at each treatment session. Six or eight MeV X-rays or ^{60}Cobalt γ-rays were used. When hemibody irradiation (HBI) was given the most affected half of the

body was irradiated first and no skin gaps were left between the upper and lower half of the body.

RESULTS

TBI was well tolerated and symptomatic side effects when they occurred were minimal. In the HBI group transient salivary gland swelling occurred in one patient, mild hair loss in three patients, subungual melanotic striae in one patient and nail fragility in one patient. White count and platelet nadirs are summarized in Fig. 1. Nadir values were reached at 4–5 weeks following the end of irradiation in the majority of patients and somewhat sooner (2–3½ weeks) for patients with bone marrow infiltration. There was little change in haemoglobin levels. As expected the most important single factor in determining the degree of neutropenia and thrombocytopenia was the initial pre-irradiation counts and it is clear that the technique may be hazardous in those patients with compromized marrow function. In two patients with positive marrows there was no evidence of infiltration after irradiation. Post-mortem examination in one and repeat bone marrow biopsy in the second failed to demonstrate lymphoma.

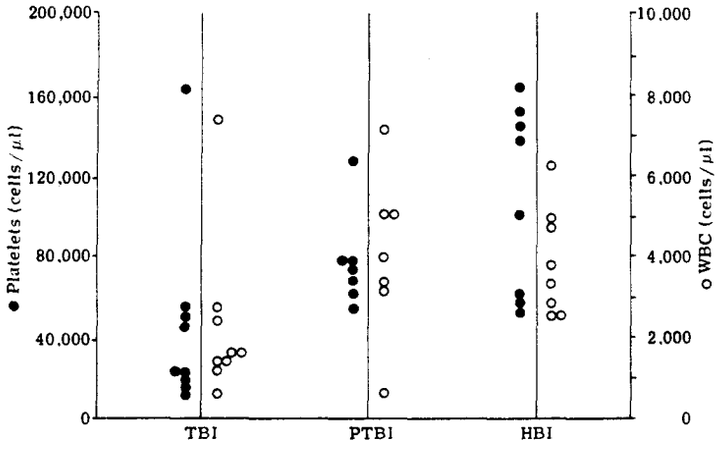


Fig. 1. Nadirs for platelets and white cells.

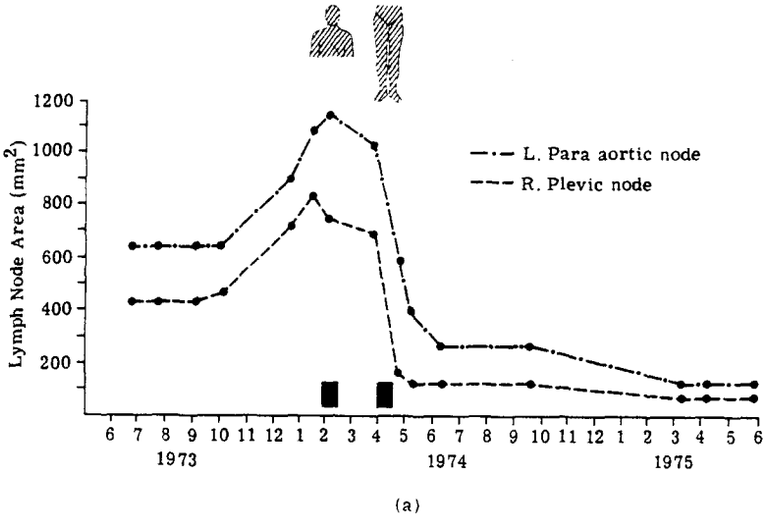
Effect on skin infiltration

Three patients had generalized skin infiltration in addition to lymph node involvement. Following TBI there was relief of pruritus and complete regression of the nodal disease in two. The majority of skin infiltrates resolved (Fig. 2), the remaining one or two patches in each patient becoming flattened and paler. One patient relapsed soon after the completion of TBI and was given systemic chemotherapy. The remaining two patients required further localized superficial irradiation to residual skin lesions; one remains in complete remission at 16.5 months.

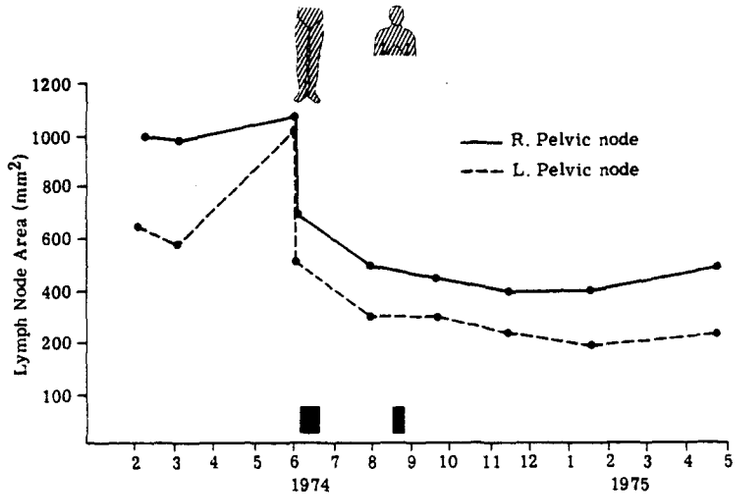
The third patient was in partial skin remission for 5.5 months following HBI. Subsequently, in relapse, one of the skin lesions was irradiated with a small local field to a total dose of 300 rad in 10 fractions over 12 days (HBI regime) with no effect. A second course of TBI was well tolerated and further regression occurred in the skin lesions. Post-mortem examination subsequently showed no evidence of bone marrow or nodal lymphoma but there was persistence of skin infiltration.

Effect on the lymph nodes

The changes in the size of involved retroperitoneal nodes opacified at lymphography were followed on sequential abdominal films by tracing the outlines of the nodes on to transparent sectional graph paper in 10 of the 24 patients. Examples of changes in nodal surface area are shown in Figs. 3 and 4. A prompt volume reduction tended to be followed by a second phase of slow regression occurring over several months (Fig. 4). An apparent abscopal effect was observed in two patients, one had complete regression of a left supraclavicular mass after the lower half of body was irradiated and in the second patient there was a reduction in size of pelvic and para-aortic nodes following upper half irradiation (Fig. 3a). Evidence of tumour progression occurring before TBI was initiated is shown in two patients with nodular lymphoma in Fig. 3 (a and b).



(a)



(b)

Fig. 3. CS III. Histology PDLN.

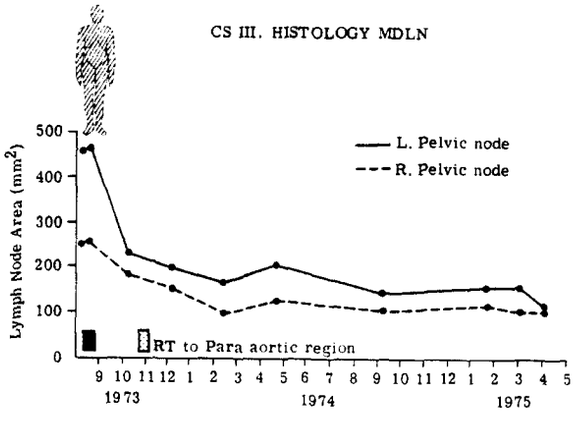


Fig. 4

Remissions

Of the 24 patients, two failed to respond to TBI. In nine of the remaining 22 patients the responses were of short duration (less than three months). In thirteen patients (54.2%) there was complete clinical and radiological regression of disease. The duration of complete remission ranged from 3.5 to 26 months with seven patients remaining in complete remission at the time of reporting (Figs. 5–7).

DISCUSSION

TBI is a simple method of treating patients with advanced non-Hodgkin's lymphoma and it is virtually unassociated with troublesome side

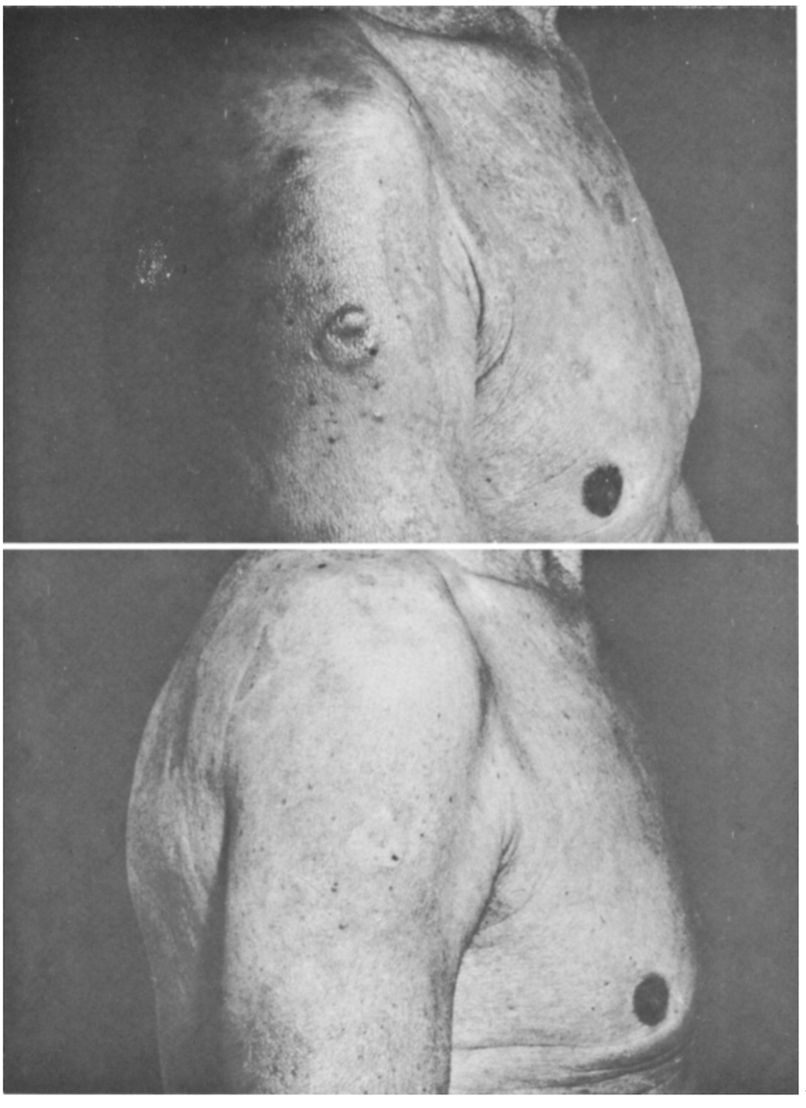


Fig. 2

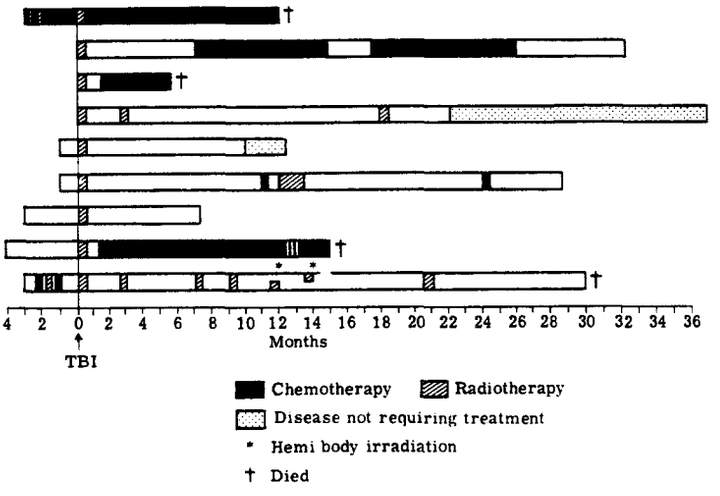


Fig. 5. *Total body irradiation.*

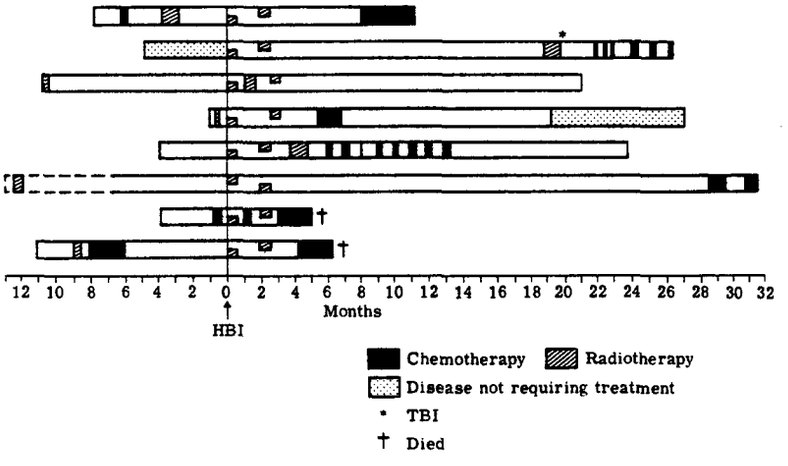


Fig. 6. *Hemi body irradiation.*

effects. The effect on the bone marrow is most pronounced in patients irradiated over a period of two weeks and is less marked when irradiation is protracted over a longer period of time or when the two halves of the body are treated sequentially, presumably because in the case of protraction bone marrow repopulation occurs between fractions and in the case of HBI the irradiated bone marrow is repopulated during the interval between the two treatments. We consider it important in patients with nodular lymphoma to observe the evolution of the tumour initially before irradiation is initiated. Adopting this approach patients with benign or slowly progressive lymphoma can be excluded. When attempting to assess the possible value of this form of low dose systemic radiotherapy it is important to bear in mind that some patients in the latter category may survive for prolonged periods with intermittent single agent chemotherapy. On the other hand the benign nature of nodular lymphoma has certainly been overstressed since 50% of patients in most series are dead by five years. Two studies have compared TBI with combination chemotherapy in lymphocytic lymphoma.

Canellos *et al.* [12] have reported the results of a randomized clinical trial comparing intensive cyclical chemotherapy (cyclophosphamide, vincristine, prednisone) and TBI in the treat-

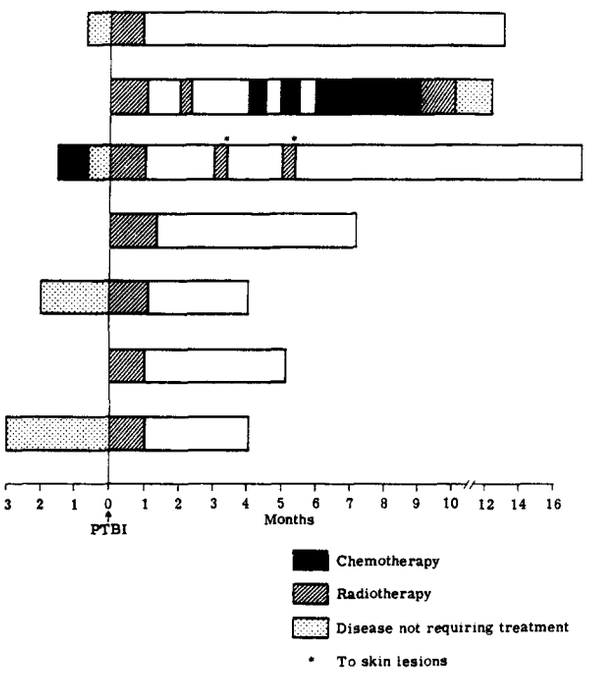


Fig. 7. Protracted total body irradiation.

ment of patients with Stage III and IV lymphocytic lymphoma. In the chemotherapy group 55% of patients achieved a complete remission compared with 56% for the irradiated group. In terms of survival there was no significant difference at three years between the two groups of patients. In a non-randomized study reported by Chaffey *et al.* [13], 80% of 25 advanced lymphocytic lymphoma patients achieved complete remission with TBI (similar to the protracted regime in the present series). In this group actual survival at two years was comparable with patients matched for age, sex and history and treated with combination chemotherapy.

The precise mechanism of action of fractionated TBI is unknown, however, there is no reason to suppose that it acts other than by a direct killing effect upon lymphoma cells since there is clearly in some patients an appreciable concomitant suppressive effect on bone marrow function. Both lymphoid cells and bone marrow stem cells show little evidence of an ability to repair damage to DNA sustained after small radiation doses. In addition to the observed marrow effects, progressive reduction in immunoglobulin levels provides evidence of an important effect of therapy on normal lymphoid function [14]. A recent experimental study demonstrates the effect of small fractions of TBI on the immune system (Davies, unpublished data).

In this experiment the clinical situation was simulated by irradiating two groups of 10 normal and 10 thymectomized CBA/Ca mice using 15 rad × 10 over 12 days to the whole body. On the third or sixtieth day following TBI, half the mice were bled and the spleen, thymus and axillary nodes were dissected and weighed. The results showed that there was up to 20% decrease in weight of the thymus and spleen at three days with slight increase of axillary lymph node weight at 60 days. Three days following TBI, leukopenia was evident in both groups, but by the sixtieth day recovery had taken place. This was more complete in the normal animals. At three days there was *ca.* 70% reduction of *T* lymphocytes which appear to be thymus independent and recovery was incomplete by the sixtieth day.

From data so far available it is clear that although a single course of TBI can produce an appreciable degree of tumour regression, relapse occurs in the majority of patients. In order to lengthen the growth delay produced by this form of treatment ways in which TBI can be integrated with chemotherapy or employed in repeated planned courses need to be explored.

It is not known whether TBI can be repeated at relatively close intervals in man. The treatment was repeated in two of our patients and produced further remissions of 2.5 and 7 months. No unexpected enhanced bone marrow toxicity was encountered. We are currently examining the feasibility of employing three courses of TBI each lasting two weeks, each course being separated by an interval of two months to allow bone marrow recovery to occur.

Acknowledgements—We are grateful to Miss Sue Moore for her help in preparing this paper.

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Resistance of Stomach Epithelium to Cytotoxic Effect of Antitumor Drugs. Increase of Sensitivity by Actinomycin D

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Abstract—*Injection of hydroxyurea (HU) resulted in degeneration after* 4 hr: *of* 94–100% *cells synthesizing DNA in small intestine crypts, of* 77% *such cells in colon epithelium and of only about* 10–15% *S-phase cells in stomach epithelium of the mouse. HU inhibited DNA synthesis in small intestine and stomach to the same extent. Thus, the ability of S-phase cells to withstand inhibition of DNA synthesis without degeneration accounts for the resistance of stomach epithelium to the cytotoxic effect of HU.*

Actinomycin D (Act D) decreased the resistance of stomach epithelium to HU. The cytotoxic effect of HU was significantly enhanced when ACT D was injected 4–16 hr *before HU. Degeneration of* 78–80% *S-phase cells in stomach epithelium was observed in these experiments. Act D injected simultaneously with HU did not increase the cytotoxic effect. Rubomycin (daunomycin) enhanced the sensitivity of stomach epithelium to HU, although to a lesser extent than did Act D.*

In animals treated with arabinosylcytosine, methotrexate, 5-fluorouracil, or phenylalanine mustard (sarcolysine) alone, the damage to colon and stomach epithelium was negligible. When Act D was injected 8 hr *before these drugs, a significant number of degenerating cells appeared in colon, but not in stomach epithelium.*

Resistance of stomach epithelium to cytotoxic effect of drugs and low sensitivity of stomach tumors to chemotherapy may have a common mechanism. The increase of cytotoxic effect of drugs by Act D or rubomycin (daunomycin) may have practical implications.

INTRODUCTION

THE TISSUE-SPECIFIC pattern of cell reaction to antitumor drugs has a significant influence on the sensitivity of tumors to chemotherapy [1, 2]. Obviously, the different sensitivity results, at least partly, from retention of some specific metabolic features of parent normal cells after malignant transformation. Therefore, some information about the mechanism of varying tumor sensitivity to chemotherapy may be obtained by studying reactions of normal tissues to chemotherapy. Also, such investigations could indicate some possibilities for increasing the tumor sensitivity.

The response of cells to inhibition of DNA synthesis is very much a function of the cell type [3]. In continuously proliferating tissues (small intestine, lymphoid tissue, tongue epithelium, erythropoietic tissue) inhibition of DNA synthesis by hydroxyurea (HU) or arabinosylcytosine (Ara-C) rapidly kill cells in the S-phase [4–7]. Cells synthesizing DNA in quiescent tissues stimulated to proliferate are resistant to the cytotoxic effect of these inhibitors.

This paper shows that cells synthesizing DNA in stomach epithelium are resistant to the damaging effect of HU, although DNA synthesis is inhibited in this tissue to the same extent as in highly sensitive epithelium of small intestine. It is estimated also that the resistance of stomach epithelium to HU can be abolished by preliminary injection of actinomycin D (Act D). This agent also enhances the sensitivity of colon epithelium to several antitumor drugs, which alone did not induce acute cell death.

MATERIAL AND METHODS

Male and female SHK albino mice were used when 7–8 weeks old. In all experiments mice received 1 μCi/g body weight of ^{3}H-thymidine (^{3}H-TDR, sp. act 20.5 Ci/mM, Moscow) injected i.p.

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HU (Calbiochem, U.S.A.), Act D (Reanal, Hungary), rubomycin (RM, antibiotic identical with daunomycin, Moscow), Ara-C (Upjohn Company, U.S.A.), methotrexate (MTX, Lederle Laboratories, U.S.A.), 5-fluorouracil (5-FU, Hoffman-LaRoche, Switzerland) and phenylalanine mustard (PAM, sarcolysine, Moscow) were dissolved in saline and administered i.p. in a volume of 10 ml/kg. The doses and schedules are indicated in the text. In all experiments the first injection was done at 10 a.m.

Mice were killed by cervical dislocation. Small intestine (a 1 cm segment beginning 1.5 cm from the pyloroduodenal junction), colon (a 1 cm segment beginning 3 cm from the anus) and stomach (dissected from the forestomach and opened along the small curvature) were removed. Tissues were fixed in Carnoy's solution and embedded in paraffin. Five μm sections were cut perpendicular to the longitudinal axis of intestine and stomach. Sections were dipped in autoradiographic emulsion (type M, Moscow), were exposed for 2 weeks and after development were stained with hemalaun–eosin.

A labeled cell was defined by the presence of at least 3 gr. The background in the autoradiographs was extremely low. Each degenerating cell counted was a round body containing condensed fragmented basophilic material, surrounded by a halo of eosinophilic cytoplasm or empty space.

The percentage of labeled degenerating cells of the whole amount of labeled cells was determined by scoring 100 degenerating and intact labeled nuclei. Degenerating non-labeled nuclei amounted to 10% or less of all degenerating nuclei and were not counted. The number of grains per cell was determined by scoring 50 labeled cells. Four mice were used in each experimental group and all data were presented as mean $\pm$ S.E.

RESULTS

Differential effect of HU on stomach and small intestine epithelium

Mice received different doses of HU 30 min after ^{3}H-TDR and the extent of cellular damage was determined in stomach and small intestine 4 hr later (Table 1). There was a significant difference in reaction of the two tissues.

Almost all cells synthesizing DNA in small intestine crypts degenerated subsequent to administration of HU in doses of 250 mg/kg or higher. This damage was a true necrogenic effect for epithelial cells and not an apparent damage resulting from destruction of intraepithelial lymphocytes. This is due to the fact that only single labeled cell or none at all, remained intact after HU. The same was observed by Farber and Baserga [6] after injection of 1500 mg/kg HU. In contrast to this, epithelial cells remained intact after administration of Ara-C [8].

Cells synthesizing DNA in stomach epithelium were resistant to the necrogenic effect of HU. Only approximately 10–15% of labeled cells degenerated after injection of 250–5000 mg/kg HU. Two doses of HU injected with a 3-hr interval also induced a weak cytotoxic effect. Thus, a 20-fold increase in the HU dose as compared to that which induced maximal damage to small intestine did not decrease the resistance of the stomach epithelium. Prolongation of HU action by administration of two doses was also ineffective.

To determine whether the observed variation

Table 1. *Effects of HU on small intestine and stomach epithelium*

HU mg/kg	Degenerating labeled cells, %† Small intestine	Degenerating labeled cells, %† Stomach	DNA synthesis‡ grains/cell Small intestine	DNA synthesis‡ grains/cell Stomach
Control	0	0	16.5 $\pm$ 0.5	22.5 $\pm$ 2.9
10	0	0	16.4 $\pm$ 0.4	22.4 $\pm$ 3.1
50	7.8 $\pm$ 3.4	0	6.7 $\pm$ 0.7	7.0 $\pm$ 0.8
250	94.0 $\pm$ 1.0	15.3 $\pm$ 5.0	0	0
500	94.0 $\pm$ 0.7	12.0 $\pm$ 1.0	0	0
1500	98.0 $\pm$ 0.7	7.3 $\pm$ 1.2	0	0
5000	98.0 $\pm$ 0.6	8.0 $\pm$ 1.2	0	0
500 × 2*	100	14.2 $\pm$ 6.4	0	0
1500 × 2*	100	14.8 $\pm$ 7.7	0	0

*Two injections with a 3 hr interval.
†^{3}H-TDR was injected 30 min before HU and mice were killed 4 hr after HU.
‡^{3}H-TDR was injected 1 hr after HU and 50 min later mice were killed.
0 is the number of grains corresponding to the background level.

in necrogenic effect of HU on stomach and small intestine resulted from different extent of DNA synthesis inhibition, mice received ^{3}H-TDR 1 hr after HU in different dose levels. As shown in Table 1, 10 mg/kg did not inhibit DNA synthesis, 50 mg/kg induced partial inhibition and 250 mg/kg induced complete (as seen in autoradiographs) inhibition in both tissues. Thus the extent of DNA synthesis inhibition by HU in small intestine and stomach was comparable, although the sensitivity of these tissues to necrogenic effect of HU was markedly different.

Increase in sensitivity of stomach epithelium to HU by Act D

To determine the effect of Act D on sensitivity of stomach epithelium to damage induced by HU, animals were given Act D (0.5 mg/kg) and, simultaneously or at intervals of 4, 8, 12 or 16 hr, they received HU (500 mg/kg). Table 2 shows that the percentage of degenerating labeled cells 4 hr after HU significantly increased when Act D was injected 4–16 hr before HU. Almost all degenerating nuclei were labeled. Administration of Act D simultaneously with HU did not enhance the cytotoxic effect. Degenerating cells were not observed in stomach epithelium 4–12 hr after injection of Act D alone.

Labeling index (LI) was determined as the number of labeled intact or degenerating nuclei per 100 μm length of epithelium. In these experiments ^{3}H-TDR was injected 30 min before HU, i.e., 3.5–15.5 hr after Act D. Since HU blocked the division of labeled cells, LI showed the number of cells synthesizing DNA at the time of HU injection. LI did not change in 4 hr after the administration of Act D and, thereafter, began to decrease (Table 2). Obviously, this resulted from blocking by Act D the G_1–S transition at the point 4–8 hr before start of DNA synthesis. Such an effect of Act D on forestomach squamous epithelium was described previously [9]. Obviously, a part of the G_1 period resistant to Act D (the so-called R-phase) exists both for stomach and for forestomach epithelium. These data indicate that, for both tissues, RNA synthesis (probably, ribosomal RNA), essential for DNA synthesis, terminates several hours before the beginning of the S-phase.

The decrease of resistance to the damaging effect of HU appeared 4 hr after the injection of Act D, at which time LI and, evidently, the composition of S-phase subpopulation were still unchanged. Thereafter the G_1–S transition was blocked which possibly lead to exhaustion of early S-phase cells.

To investigate the role of continuous DNA synthesis in the decrease of resistance to HU, mice were injected twice with HU, the first time simultaneously with Act D (Exp. No. 8, Table 2). ^{3}H-TDR was given 4 hr after the first injection, when partial restoration of DNA synthesis occurred. Comparison of experiments No. 4 and No. 8 shows that additional injection of HU did not change the number of degenerating cells. This indicates that continuous DNA synthesis after Act D was not necessary for enhancement of the sensitivity to HU.

Table 2. Increase in sensitivity of stomach epithelium to HU by Act D and RM (daunomycin)

Exp. No.	Drugs and schedule*	Degenerating labeled cells, %†	LI‡
1.	HU	16.2±2.5	8.8±0.4
2.	Act D	0	—
3.	Act D+HU	23.0±2.6	9.1±1.1
4.	Act D−4 hr−HU	78.0±1.9	8.9±1.0
5.	Act D−8 hr−HU	77.5±0.9	5.9±0.3
6.	Act D−12 hr−HU	91.8±2.4	4.5±0.7
7.	Act D−16 hr−HU	91.3±2.4	2.3±0.4
8.	Act D+HU−4 hr−HU	85.5±2.5	—
9.	Act D+HU−4 hr−HU	67.5±10.6	—
10.	Act D−8 hr−HU	29.3±4.5	—
11.	RM	0	—
12.	RM+HU	24.8±1.7	—
13.	RM−8 hr−HU	53.8±7.8	—

*Animals were treated with 500 mg/kg HU, 0.5 mg/kg Act D and 4 mg/kg RM.

†^{3}H-TDR was injected: 30 min before HU in Exp. No. 1, 3–7, 12, 13; 30 min before second injection of HU in Exp. No. 8, and 30 min before Act D or RM in Exp. 2, 9–11. Mice were killed 4 hr after last injection of HU (Exp. 1, 3–10, 12, 13) or 4–12 hr after Act D and RM (exp. 2, 11).

‡Number of labeled nuclei per 100 μm length of epithelium.

In experiments Nos. 9 and 10, as distinct from others, ^{3}H-TDR was injected 30 min before Act D. When HU was injected twice to inhibit the exit of labeled cells from the S-phase, the number of degenerating labeled cells was high. When HU was injected 8 hr after Act D the number of degenerating cells decreased. This evidently was a consequence of exit of labeled cells from the S-phase by the time of HU injection.

To estimate the possibility that not only Act D, but also other drugs inhibiting RNA synthesis could enhance the sensitivity of stomach epithelium to HU, rubomycin (antibiotic identical with daunomycin) was used. Injection of rubomycin 8 hr before HU significantly increased the number of degenerating labeled cells, although to a lesser extent than did Act D (Table 2).

Effect of antitumor drugs on stomach and colon epithelium

On the basis of the data derived from the preceding experiments it seemed reasonable to investigate the effect of Act D on the sensitivity of epithelium to antitumor drugs other than HU.

The colon instead of the small intestine was investigated, because after injection of Act D alone cellular damage was observed in the small intestine but not in the colon. Also, PAM, 5-FU and Ara-C which alone induced negligible damage in colon led to appearance of significant number of karyorrhectic cells in small intestine. Obviously, the damage observed in small intestine resulted, at least in part, from necrosis of intraepithelial lymphocytes [8]. It may be suggested that the content of such lymphocytes in colon epithelium is much lower.

As shown in Table 3 colon epithelium was significantly more sensitive to HU than was the stomach epithelium. Thus the type of reaction to HU in colon was similar to that in small intestine as in both tissues the majority of S-phase cells degenerated.

In animals treated with Ara-C, 5-FU, PAM or MTX alone there were no or only single karyorrhectic cells in stomach and colon. When Act D was injected 8 hr before these drugs, a significant number of degenerating labeled cells appeared in colon epithelium but not in stomach epithelium (Table 3).

Thus colon epithelium appeared to be significantly more sensitive than stomach epithelium to the cytotoxic effect of drugs. Whereas sequential administration of Act D and HU was the only schedule at which a significant damage to stomach epithelium was induced, in the colon epithelium an abundance of degenerating cells appeared upon injection of HU alone or several drugs that were injected after Act D.

DISCUSSION

The mechanism of acute cell death induced in proliferating tissues by HU and other drugs does not seem to be quite clear. Prevention of acute cell death by inhibitors of protein synthesis indicates involvement of protein synthesis in this form of damage [7, 10, 11]. On the basis of these data acute cell death is considered as active reaction dependent upon new protein formation, possibly, enzyme induction [10].

The results of the present study are in agreement with this suggestion. Inhibition of RNA synthesis by Act D frequently induces an increase in the concentration (activity) of a specific protein (phenomenon of "superinduction") [12, 13]. Possibly, administration of Act D and RM lead to superinduction of a protein which is necessary for appearance of acute cell death in stomach epithelium. This effect was not specific for HU, as Act D increased the sensitivity of colon epithelium to 5-FU, MTX, PAM and Ara-C. The protective effect of

Table 3. Effect of antitumor drugs on stomach and colon epithelium

Drug	Dose mg/kg	Degenerating labeled cells, %*			
		Drug alone		Act D (0.5 mg/kg) – 8 hr – Drug	
		Stomach	Colon	Stomach	Colon
HU	500	16.2 ± 2.5	76.3 ± 4.8	77.5 ± 0.9	90.0 ± 3.8
Ara-C	50	4.2 ± 2.3	3.8 ± 1.0	18.5 ± 2.8	34.0 ± 4.3
MTX	50	0	4.3 ± 2.4	0	23.0 ± 3.9
5-FU	200	0	2.0 ± 0.9	9.2 ± 4.0	42.7 ± 1.7
PAM	4	0	0	6.3 ± 2.5	55.2 ± 10.5

*^{3}H-TDR was injected 30 min before HU, ara-C, MTX, 5-FU and PAM.

cyclohexemide also was not specific for inhibitors of DNA synthesis, as the cytotoxic effects of nitrogen mustard and X-rays also were diminished by this inhibitor [10]. Thus, Act D has an effect on some general mechanisms determining cell death.

Absence of an enhancing effect of Act D injected simultaneously with HU corresponds to superinduction as a mechanism of increase in sensitivity. Also in favour of this hypothesis is the nature of acute cell death, i.e. complete destruction of nuclei within 2–4 hr. This effect probably needs the contribution of enzyme activity.

The relation between acute cell death and loss of clonogenic activity (reproductive death), which is the most important effect of antitumor drugs, remains to be established. Obviously, not all the cells which lost clonogenic activity appeared within hours as karyorrhectic bodies. There are many examples of reproductive cell death without signs of acute cell damage. But there exist some common features between two types of cell death. Both types of cell death are prevented by cyclohexemide [10, 14]. Some parallelism exists between the sensitivity of cells to two types of damage. Human tumors of lymphatic origin are known to be highly responsive to chemotherapy [15], and normal lymphatic tissue is most sensitive to acute damage by metabolic inhibitors [3]. On the other hand, stomach tumors are resistant to almost all drugs, and normal stomach epithelium shows low sensitivity to the cytotoxic effect of drugs. Therefore, it seems possible that drug schedules which increase the sensitivity of cells to the acute damaging effect of drugs may also decrease the resistance of tumors to chemotherapeutic agents.

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Glycine-N-Methyltransferase Levels in Human Breast Cancer Tissue

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Abstract—*The activity of glycine N methyltransferase was studied in 156 human mammary carcinomas, 24 metastatic ganglions and 46 benign breast tumors. This activity is higher than that of normal mammary tissue. The role of this enzyme in the regulation of the transfer RNA methylation in human mammary carcinoma is discussed*

INTRODUCTION

THE TRANSFER RNA methyltransferases are enzymes which modify the structure of preformed transfer RNA (t. RNA), by the insertion of methyl group into specific positions in the four main bases of t. RNA. These enzymes are species specific, organ specific and base specific. Alterations in the t. RNA methylating enzymes implicate not simply the specific activity of the enzymes, but also qualitative changes.

It was suggested that carcinogenesis was linked to a modification of the methylation of nucleic acids. In mammary carcinoma cells of a mouse strain, Turkington *et al.* [1] found 4 differences in the t. RNA methyl transferases activities when compared with normal mammary epithelial cells: the activity of the t. RNA methyltransferases in mammary carcinoma cells is higher than that of normal mammary cells, the intracellular content of t. RNA is not modified, the alterations are observed only for some enzymes such as uridine 5 methylase and often the appearance of new enzymes is associated. Borek *et al.* [2] reported hypermethylated t. RNA in tumor tissues whereas Randerath [3] described the undermethylation of t. RNA in some hepatomas: 7777 and 5123 D.

Some natural inhibitors of t. RNA methylating enzymes were found in crude extracts of adult animal tissues and they were implicated in *in vivo* regulation of these enzymes. One of them was obtained from rabbit or rat livers [4], it was resolved into 2 fractions, a high mol. wt protein and a low mol. wt compound. These two components were identified as an enzyme system which N-methylates glycine to yield sarcosine and S-adenosylhomocysteine [5]. Glycine-N-methyltransferase (GNMT) competes with the t. RNA methyltransferases, for the methyl donor, S-adenosylmethionine. The product of the reaction, S adenosylhomocysteine (the low mol. wt compound) is a potent inhibitor of the t. RNA methyltransferases.

It was suggested that this enzyme system regulates the t. RNA methyltransferases levels since an inverse relationship was shown between t. RNA methyltransferases and glycine-N-methyltransferase [6]. The levels of GNMT activity were high in adult tissues and very low in fetal and tumor tissues whose t. RNA methyltransferases exhibited elevated levels.

These studies were performed in fetal rabbit liver and hepatoma. No results about human mammary carcinoma glycine-N-methyltransferase were reported and the objective of our investigations was to determine whether this enzyme is a regulating system of the t. RNA methyltransferases which exhibited high levels in mammary tumors [7].

MATERIAL AND METHODS

Neoplastic and non neoplastic human mammary tissues were obtained during mastectomy or biopsy, and normal parenchymal mammary tissue was derived from cancer-free portions of the resected breast. Samples were trimmed of fat tissue, weighed and immediately treated for determination of glycine-N-methyltransferase.

Histopathological examination of samples pointed out their properties related to differentiation, mitoses, nuclear morphology, which reflect the degree of cancer aggressiveness (classes I, II and III).

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Tissues were homogenized in a buffer containing Tris–HCl buffer 10 mM, pH 7.4, NaCl 10 mM and $MgCl_2$ 1.5 mM. The homogenate was centrifuged successively for 10 min at 30,000 ***g*** and 60 min at 105,000 ***g***.

Glycine-N-methyltransferase was tested on the high speed supernatant (0.1 ml) according to Heady *et al.* [8]. The assay mixture contains 50 mM Tris–HCl buffer, 1 mM dithiothreitol, 12.5 μM S. adenosyl [Me–^{14}C] methionine (45 mCi/mM), 10 mM glycine, in a total volume of 0.2 ml. After incubation at 37°C for 30 min, the reaction is stopped by addition of 0.050 ml of 20% phosphotungstic acid. Non radioactive S. adenosyl methionine was added to precipitate S-adenosyl [Me–^{14}C] methionine. The reaction mixture is then made up to 1 ml with cold distilled water and is centrifuged at 30,000 ***g*** for 10 min. A 0.1 ml aliquot of the supernatant is added to 10 ml Instagel (Packard) and counted in a liquid scintillation system (Intertechnique).

A unit is the amount of enzyme which gives 1 nmole of sarcosine in 30 min. The determination of protein is performed by the method of Lowry [9].

Sarcosine was characterized by liquid chromatography with isopropanol–water-formic acid, 80–20–4, where it gave a ninhydrin positive spot.

RESULTS

Intracellular glycine-N-methyltransferase levels in tissue samples are summarized in Table 1. The average level of GNMT specific activity obtained from 156 malignant breast tissues is 455 pmole/mg protein. As it can be seen, this value is significantly higher than in normal breast tissues (the average level is 214 pmole/mg protein). Similar results were observed with metastatic ganglions. In benign tumors (proliferative dysplasias and fibroadenomas) specific activity of GNMT is in the normal range.

These last results (benign tumors GNMT differs significantly with that of malignant tissues: $P<0.001$) eliminate criticisms about the different cell populations present in different proportions in the tissues analyzed. It is known that malignant and benign tissues contain more epithelial cells than normal mammary tissues. Therefore differences observed here are bound to malignity and not to the proportion of epithelial cells.

In Table 2, results are expressed according to increasing enzymatic activity and it can be seen that distribution is statistically different between malignant and normal breast tissues, and between metastatic ganglions and benign breast tumors.

No significant relationship was found between GNMT levels and histological type of tissues.

DISCUSSION

Our results obtained in human neoplastic breast tissues appear contradictory to the idea that glycine N-methyltransferase can regulate the activity of the t. RNA methyl transferases both by competition for S-adenosylmethionine and by the generation of the inhibitory product S-adenosylhomocysteine.

This hypothesis was confirmed by Heady [6] who reported an inverse relation between t. RNA methyltransferase activity and glycine-N-methyltransferase activity which is very low in hepatomas.

In mammary carcinoma cells of the C3H mouse total t. RNA methyltransferases level was described higher than that of normal mammary cells [7], and it would be logical to have low levels of glycine-N-methyltransferase in human mammary carcinoma if this enzyme regulates the t. RNA methyltransferases in this tumor.

However, all these data were obtained *in vitro* in an artificial environment outside the regulatory controls imposed by tissue organization.

Yet, Turkington [10] described an increase of

Table 1. *Glycine-N-methyltransferase in nonneoplastic and neoplastic human mammary tissues*

Tissues	Samples	GNMT pmole mg protein/30 min	P*
Normal breast tissues	69	214 ± 37	
Benign breast tumors	46	282 ± 72	N.S.
Malignant breast tissues	156	455 ± 78	$<1.10^{-6}$
Metastatic ganglions	24	534 ± 235	$<1.10^{-4}$

The values are expressed as the mean ± S.E.M.
*Compared with normal breast tissue.

Table 2. Distribution of glycine-N-methyltransferase activity in nonneoplastic and neoplastic human mammary

GNMT activity pmole/mg protein/30 min	Normal breast tissues	Benign breast tumors	Malignant* breast tumors	Metastatic* ganglions
0–100	20	7	11	1
101–200	20	14	17	5
201–300	8	12	38	4
301–400	14	5	36	4
401–500	3	4	14	0
501–1000	4	2	31	8
>1000	0	2	9	2

The results are expressed as the number of samples in each category.
*Significantly different from normal breast tissue (Xr test) $\alpha = 1\%$.

t. RNA synthesis and t. RNA methyltransferases activity in mouse mammary gland under the influence of hormones such as insulin, prolactin, and hydrocortisone, during pregnancy. t. RNA methyltransferase activity in rat uterus is under the dependance of oestrogens [11].

According to our results, it seems unlikely that glycine-N-methyltransferase is a regulating system for the t. RNA methyltransferases activity in human mammary carcinoma, and it is quite possible that the t. RNA methyltransferases are under the influence of hormones such as oestradiol which is known to regulate the growth of a great many breast tumors.

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Enzyme Studies in Human Breast Tumours

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Abstract—*The activities of 7 enzymes associated with glycolysis were measured in both carcinomas and non-malignant breast tissues. The results were analysed in three different ways, i.e. according to tissue weight and enzyme/LDH and enzyme/6PGDH ratios. The following differences were observed.*

1. *The carcinomas showed higher enzyme activities than the non-malignant mammary tissues.*
2. *There were no differences between enzyme activities in primary carcinomas from patients with stage* I, II, III + IV *disease and metastatic tissue, indicating that enzyme activities in primary tumours are unrelated to the spread of the disease.*
3. *The analysis of results according to the malignancy grade of the carcinomas showed that there were no significant differences in enzyme activities between grades* I *and* II. *The grade* III *tumours showed higher activities for 6PGDH, PGM, LDH and PHI.*
4. *The enzyme patterns showed that the ratio of LDH to PFK, 6PGDH and α-GPDH were lower in carcinomas than in non-malignant tissues. Similarly, the ratio of 6PGDH to PGM and α-GPDH were also significantly lower in carcinomas.*
5. *Analyses of tumour enzyme ratios according to the stage of the disease showed no significant differences between stages* I *to* IV, *but when the same tumours were classified by malignancy grade, there was a gradual decrease in G6PDH/6PGDH and α-GPDH/6PGDH from grades* I *to* III.
6. *The significance of these findings is discussed in terms of alterations in the balance between metabolic pathways.*

INTRODUCTION

A MAJOR portion of research in human breast cancer is directed towards a search for biochemical parameters which will assist in predicting the clinical course of the disease. The studies on Morris and Novikoff hepatomas have shown that the growth rates of these animal tumours are related to the enzymes associated with carbohydrate metabolism [1]. Attempts have been made to form a similar concept for human breast tumours on the basis of similarities in enzyme patterns between these neoplasms and animal tumours whose growth rates and hormonal dependence are well established [2, 3]. It was therefore decided to test whether there is any correlation between enzyme patterns and the clinical course of the disease. This paper reports on these patterns in primary carcinomas of the breast on the basis of pathological staging and grading.

MATERIAL AND METHODS

All the chemicals used in the study were purchased from British Drug Houses Ltd. and were of analytical grade. α-GPDH, TPI, G6PDH, aldolase, NADH, $NADP^+$, ATP, F-6-P, G-6-P, 6PG and pyruvate were purchased from Boehringer Mannheim. G-1-P was purchased from Koch-Light Ltd. DHAP was prepared from the dicyclohexylammonium salt of the dimethylketal according to the manufacturer's (Boehringer Mannheim) instructions.‡

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‡The following abbreviations are used in this paper.

Enzymes

α-GPDH, α-glycerolphosphate dehydrogenase; TPI, triosephosphate isomerase; G6PDH, glucose-6-phosphate dehydrogenase; PFK, phosphofructokinase; 6PGDH, 6-phosphogluconate dehydrogenase; PGM, phosphoglucomutase; LDH, lactate dehydrogenase; PHI, phosphohexose isomerase.

Co-enzymes

NADH, reduced nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate.

Substrates

F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; 6PG, 6-phosphogluconate; G-1-P, glucose-1-phosphate; DHAP, dihydroxyacetone phosphate.

Both malignant and non-malignant tissues were obtained from patients undergoing breast surgery. Non-malignant tissues were obtained from 17 patients with benign mammary dysplasia (BMD) and 4 undergoing reduction mammoplasty (RM) and from patients with fibroadenomas. As there were no statistically significant differences between BMD and RM in the enzyme activities the results were combined. Tissues from patients with benign mammary dysplasia included fibrocystic disease and fibroadenosis. Only involved tissues were assayed. No tissue with a marked epithelial hyperplasia was included as they were all processed for histological examination. Malignant tissue came from primary carcinomas and metastatic tumours in lymph nodes and skin. The tissues were classified by histological examination and a small piece (400 mg) adjacent to the one taken for histology was wrapped in aluminium foil, frozen on solid carbon dioxide and transferred to the laboratory where it remained frozen ($-20°C$) until further processing. Tumours were staged on pathological data according to the TNM classification [4]. The carcinomas were graded by the criteria of Bloom & Richardson [5]. This is based on the degree of tumour differentiation, the pleomorphism of nuclei, the number of hyperchromatic nuclei and mitotic figures. Grade I tumours are of lower malignancy and better differentiated than grade II tumours and grade III tumours are the most malignant.

The tissues were cleared of any surrounding fat, weighed and cut into small pieces. The semi-frozen tissues were then homogenized in a Silverson homogenizer according to the procedure of Shonk & Boxer [6]. The homogenates were centrifuged (800 ***g*** for 15 min) at 4°C, the cytosol faction was decanted off and used as a source of enzymes.

A 5% solution for malignant and 10% for non-cancerous tissues were used in these estimations. The activities of PFK, G6PDH, 6PGDH, PGM, LDH, α-GPDH and PHI were measured in all tissues. Enzyme assays were performed in a Beckman recording spectrophotometer according to the procedure of Shonk and Boxer [6] with the exception that 1 min readings were recorded automatically for 5 min and a mean of the last 4 was taken as an average change in optical density.

The results were calculated on the basis of units (U) of enzyme activity per gram wet weight of tissue. A unit is defined as that amount of enzyme which will catalyze the transformation of 1 μmole of substrate per minute. The results were analyzed in 3 different ways: as units of enzyme activity/g and as enzyme/LDH and enzyme/6PGDH ratios. They are presented as mean ± S.D. The means were tested for significance by a Student's *t*-test.

RESULTS

In this study the enzyme activities were estimated in 21 patients with BMD + RM, 24 with fibroadenomas, 125 with primary tumours and 25 from whom metastatic tumour was obtained.

The values (U/g) obtained in BMD + RM, fibroadenomas, stage I–IV cancers and metastases are shown in Table 1.

There is a significant increase in 6PGDH, LDH, PHI and a decrease in α-GPDH between BMD + RM and fibroadenomas. When values obtained for fibroadenomas were compared with stage I cancers, significantly higher values were obtained for all the enzymes in cancerous tissues. Similarly, comparison between BMD + RM and stage I carcinomas shows that, with the exception of α-GPDH, all the enzyme activities were higher in the carcinomas.

The differences between the malignant tissues were then evaluated. There were 53 patients with stage I, 49 with stage II, 23 with stages III and IV disease. No significant differences were found between stages I and II or between primary tumours from stages III + IV and metastatic tissue. When values from stage II carcinomas were compared with stages III + IV, significantly higher values were observed in the latter for PFK and PHI. Similarly when results from stage I primaries were compared with those from metastatic tissues significantly higher values were found in the metastatic tissues for G6PDH, 6PGDH and PHI.

The tumours were then separated on the basis of malignancy grade [5]. Table 2 shows the results expressed as enzyme activity per gram of tissue. There were no significant differences between grades I and II. 6PGDH and LDH activities were significantly higher in grade III compared to grade II. Similarly, higher activities for 6PGDH, PGM, LDH and PHI were observed when grade III tumours were compared with grade I.

The results shown in Table 1 for non-malignant tissues and stage I carcinomas were then re-analyzed as enzyme/LDH and enzyme/6PGDH ratios. The results which showed statistically significant differences are shown in Table 3.

Significant differences were observed between BMD + RM and fibroadenomas for both

Table 1. Enzyme activities (μmole of pyridine nucleotide reduced or oxidized/min/g tissue) in human breast tissues

	BMD + RM	Fibroadenoma	Stage I	Stage II	Stages III + IV	Metastases
PFK	0.016 ± 0.016 (18)	0.028 ± 0.029 (23)‡	0.060 ± 0.045 (48)†	0.067 ± 0.040 (43)	0.119 ± 0.075 (22)§	0.078 ± 0.065 (19)
G6PDH	0.036 ± 0.027 (18)	0.040 ± 0.029 (15)‡	0.133 ± 0.085 (47)†	0.186 ± 0.117 (47)	0.226 ± 0.161 (23)	0.252 ± 0.163 (19)
6PGDH	0.023 ± 0.017 (21)*	0.035 ± 0.013 (17)‡	0.132 ± 0.093 (52)†	0.136 ± 0.066 (49)	0.144 ± 0.078 (23)	0.162 ± 0.087 (21)‖
PGM	0.358 ± 0.227 (20)	0.553 ± 0.405 (23)‡	1.327 ± 0.763 (53)†	1.480 ± 0.717 (49)	1.383 ± 0.754 (22)	1.802 ± 0.729 (21)
LDH	1.068 ± 0.629 (21)*	1.742 ± 0.946 (24)‡	6.381 ± 3.460 (51)†	7.087 ± 3.411 (45)	8.963 ± 4.678 (23)	8.478 ± 4.100 (25)
α-GPDH	0.156 ± 0.169 (21)*	0.028 ± 0.019 (19)‡	0.173 ± 0.121 (40)	0.196 ± 0.137 (47)	0.226 ± 0.202 (22)	0.168 ± 0.102 (21)
PHI	1.525 ± 0.698 (21)*	2.030 ± 0.759 (12)‡	7.421 ± 3.124 (33)†	7.303 ± 3.420 (29)	10.903 ± 5.811 (20)§	10.233 ± 5.079 (16)‖

The results are expressed as mean ± S.D. The numbers in parentheses indicate the number of estimations. BMD, Benign Mammary Dysplasia; RM, Reduction Mammoplasty; stages I–IV, Primary Tumours. There were no significant differences between stages I and II or between stages III + IV and metastases. *P* values of less than 0.02 are classed as significant.

*Significant differences between BMD + RM and fibroadenomas.

†Significant differences between BMD + RM and stage I.

‡Significant differences between fibroadenomas and stage I.

§Significant differences between stages II and III + IV.

‖Significant differences between stage I and metastases.

Table 2. Enzyme activities (μmole of pyridine nucleotide reduced or oxidized/min/g tissue) in human breast tumours

	Grade I	Grade II	Grade III
PFK	0.056 ± 0.038 (13)	0.078 ± 0.061 (45)	0.111 ± 0.097 (39)
G6PDH	0.136 ± 0.080 (12)	0.200 ± 0.111 (43)	0.212 ± 0.181 (39)
6PGDH	0.083 ± 0.052 (12)	0.120 ± 0.064 (43)	0.163 ± 0.084 (39)**
PGM	1.030 ± 0.502 (15)	1.376 ± 0.792 (46)	1.707 ± 0.735 (38)**
LDH	5.094 ± 3.128 (16)	7.463 ± 4.113 (45)¶	10.131 ± 4.805 (38)**
α-GPDH	0.167 ± 0.099 (15)	0.241 ± 0.213 (43)	0.181 ± 0.120 (35)
PHI	6.214 ± 2.895 (11)	8.254 ± 3.967 (25)	9.937 ± 3.903 (25)

The tumours were graded according to the classification of Bloom and Richardson (1975). Results are expressed as mean ± S.D. The numbers in parentheses indicate number of estimations. *P* values of less than 0.02 are classed as significant.

¶Significant differences between grades II and III.

**Significant differences between grades I and III.

Table 3. The ratios of various enzymes to lactate dehydrogenase (LDH) and 6-phosphogluconate dehydrogenase (6PGDH) in human breast tissues

	Enzyme/LDH						Enzyme/6PGDH					
	BMD + RM		Fibroadenoma		Stage I		BMD + RM		Fibroadenoma		Stage I	
PFK	0.014 ± 0.007	(18)	0.019 ± 0.018	(24)‡	0.010 ± 0.006	(52)†	0.711 ± 0.459	(18)	0.502 ± 0.270	(19)	0.625 ± 0.446	(50)
6PGDH	0.021 ± 0.006	(21)	0.025 ± 0.013	(19)‡	0.017 ± 0.006	(45)†	—		—		—	
PGM	0.323 ± 0.162	(18)	0.261 ± 0.188	(23)	0.196 ± 0.087	(51)†	16.354 ± 8,519	(19)	13.729 ± 6.414	(20)	11.318 ± 4.589	(49)†
α-GPDH	0.154 ± 0.100	(21)*	0.033 ± 0.029	(21)	0.030 ± 0.023	(39)†	6.773 ± 4.271	(19)*	1.479 ± 2.306	(18)	3.413 ± 4.350	(48)†

The results are expressed as mean ± S.D. The numbers in parentheses indicate number of estimations.
BMD, Benign Mammary Dysplasia; RM, Reduction Mammoplasty; stage I, stage I Primary Tumour. *P* values of less than 0.02 were classed as significant.
*Significant differences between BMD + RM and Fibroadenomas.
†Significant differences between BMD + RM and stage I.
‡Significant differences between Fibroadenomas and stage I.

Table 4. The ratios of G6PDH and α-GPDH to 6PGDH in human breast tumours

Enzyme/6PGDH	Grade I		Grade II		Grade III	
G6PDH	1.638 ± 0.743	(12)	1.748 ± 0.863	(42)§	1.088 ± 0.522	(36)
α-GPDH	2.718 ± 2.123	(12)	1.937 ± 1.484	(39)	1.365 ± 1.087	(36)‖

The tumours were graded according to the classification of Bloom and Richardson (1957).
Numbers in parentheses indicate number of estimations. *P* values of less than 0.02 were taken as significant.
§Significant differences between grades II and III.
‖Significant differences between grades I and III.

α-GPDH ratios. When the results for BMD + RM and stage I cancers were compared, both ratios yielded significant differences for α-GPDH and for PGM. Only LDH ratios gave significantly different results for PFK and 6PGDH when stage I cancers were compared with either BMD + RM or fibroadenomas.

The ratios for malignant tissues were then analyzed to test for significance between stages I–IV. There was no significant difference between the stages for either the LDH or the 6PGDH ratios.

When tumours were separated according to grade, there were no significant differences for the LDH ratios. However, the 6PGDH ratios showed some differences between the grades. G6PDH ratios were significantly higher in grade II than in grade III. Similarly, the α-GPDH ratio was higher in grade I than in grade III (Table 4).

DISCUSSION

The technical problems associated with the storage, homogenization of tumours and the presentation of results have been discussed in detail in a review article by Shonk and Boxer [7]. In this study we have followed their suggestions as far as is possible. Of the parameters available, i.e. DNA, RNA, proteins, tissue wet weight and ratios of enzymes, the reviewers suggested that on balance, measurement of gram wet weight of tissue is easy and facilitates the comparison of results obtained in various laboratories. Alternatively, the use of enzyme ratios minimizes within tumour differences due to methodological variations, thus making the comparison between tumours more reliable. In this study, therefore, we have used both these parameters to analyze the results.

Comparisons between the data in this paper and that published by other workers show certain similarities. Four of the enzymes assayed by Smith, King, Meggitt and Allen [8] are also reported here but their results are expressed in terms of DNA measurements. If they are converted to enzyme/LDH ratios, they are within the range of values reported here. LDH activities in our study are significantly lower than those reported by Goldman, Kaplan and Hall [9] and PHI levels similar to those observed by others [8, 10, 11].

Hilf *et al.* [11] have reported on the activities of various enzymes in normal breast tissue, fibrocystic disease and infiltrating ductal carcinoma and found significant differences between cancerous and normal tissues. The results in Table 1 are in general agreement in that the activities in tumours are higher than those found in BMD + RM or fibroadenomas. However, our results show lower activities per gram weight for G6PDH and α-GPDH and higher values for PGM in these tissues. Furthermore, their values fluctuate within a narrow range whereas we have found considerable variations within these categories. The differences may be partially due to the methodological compromises required to enable such a number of enzyme activities to be assayed in a single tissue sample.

Lower enzyme activities on a weight basis may simply reflect differences in the cellularities of various tissues and do not necessarily show changes in enzyme patterns. However, the rise in LDH activity appears to be disproportionately high, a property which human breast carcinomas seem to share with other malignant tissues [12]. The reason for this high lactate production is not known but could be explained either on the basis of mitochondrial inability to oxidize pyruvate efficiently by way of the citric acid cycle enzymes or there is a change in the mechanism(s) regulating glycolysis. Lower α-GPDH activity coupled with high lactate production found in our study and reported by others for animal tumours [13] has resulted in the lowering of α-GPDH/LDH, which is one of the most significant metabolic differences between normal and malignant cells. This phenomenon indicates that lipids in neoplastic cells are channelled into the pathways of carbohydrate metabolism rather than fat deposition. The activity of PFK has been found to be a limiting factor in glycolysis in both HeLa cells and Ascites tumour cells [14, 15]. If the rate of glycolysis is correlated with the growth rate in human breast carcinomas, as has been demonstrated in hepatomas [1], one might expect this to be closely reflected in PFK activities. This was not borne out by the results presented here. Therefore, it would seem that, if any change does occur, it is likely to happen prior to the stage at which a benign condition requires surgical investigation. The activities of G6PDH either on a weight basis or as enzyme ratios showed no major differences between various categories of tumours suggesting that the hexose monophosphate pathway is unaffected by progression of the disease. PGM levels showed a gradual rise between BMD + RM and stage I carcinomas and when the tumours were separated according to grade, suggesting that the degree of potential malignancy may correlate with the rate of glycogen metabolism.

If the concept outlined by Hilf *et al.* [3] is valid and it is possible to employ enzyme patterns to predict the clinical course of the disease a new

and useful parameter will be available to the clinician. Therefore we have analyzed our results on the basis of currently known parameters such as stage of the disease or grade of the tumour. Our data clearly indicates that the presence or otherwise of metastases is unrelated to the enzyme patterns in primary tumours as there are no significant differences between stages I–IV. This is in agreement with the work of Silvestrini *et al.* [16] who have reported that the presence of metastases at the time of mastectomy has no effect on the proliferation rate of the primary tumour. Furthermore, the same authors [16] reported that there are no significant differences between primary tumours and involved lymph nodes with regard to proliferation rates; this was also true for the enzyme levels reported here. Thus, the spread of the disease, as measured by clinical staging, does not influence the enzyme activities in the primary tumours.

Significant differences were observed in the activities of 6PGDH, PGM, LDH and PHI when the tumours were separated on the basis of pathological grades which are representative of the potential malignancy of the neoplasm. Muir and Fawcett [10] measured PHI activities in breast tumours and reported a gradual rise from grades I to III. Our results are in agreement with their findings. However, they have reported even higher values for fibroadenomas which we are unable to confirm. When the ratios, enzymes/LDH and enzyme/6PGDH, were compared the LDH ratios showed no differences between the grades but 6PGDH ratios for both G6PDH and α-GPDH were significantly different. In particular the α-GPDH ratio showed a gradual decline as the potential malignancy of the tumour increased. Whether this represents a genuine difference in energy requirements remains to be seen.

In conclusion, analysis of data on the basis of these parameters showed some differences in enzyme activities in primary breast tumours. However, this approach in which the patients are grouped together on the basis of one parameter can only show differences when a profound change has occurred. It remains to be seen whether they correlate with either the recurrence rates or responsiveness to endocrine treatment on an individual basis.

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Further Studies on the Differences in Serum Dependence in EBV Negative Lymphoma Lines and Their *In Vitro* EBV Converted, Virus-Genome Carrying Sublines*

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Abstract—*Independently EBV-converted, viral genome carrying sublines of the originally EBV negative Ramos and BJAB lymphoma lines showed decreased serum dependence, in comparison with the progenitor lines. The virus negative lines could not grow in* 10% *dialysed FCS, unless reconstituted with the dialysate. The converted lines grew well on dialysed serum. Seeding of the EBV negative lines with larger inocula enabled them to "take off" on dialysed serum as well. The EBV converted lines were able to form colonies in soft agar, whereas the original negative lines failed to do so.*

INTRODUCTION

LYMPHOBLASTOID cell lines can be readily established by infecting cord blood lymphocytes with Epstein–Barr virus (EBV) or by explanting lymphocytes from EBV seropositive donors. EBV-carrying African Burkitt's lymphoma biopsies are also readily established as lines, known to represent the neoplastic cell itself in most cases. Lines of all these types contain EBNA and EBV–DNA. It is relatively easy to maintain EBV positive cell lines *in vitro*. In contrast, there are very few EBV negative lines with B cell characteristics. Their maintenance is far more difficult [1].

Two EBNA negative human lymphoma lines (Ramos and BJAB) have been converted to several EBV-carrying sublines by infecting them with two EBV strains (B958 and RP3HR1) [2, 3]. Briefly, P3HR1 and B958 supernatants were added to Ramos and BJAB cells, and then cultured. The cells were allowed to grow with regular feeding, and the EBNA was followed. In some of the cultures all nuclei became positive for EBNA and this characteristic was retained. Only these cultures at that stage were considered as EBV converted sublines. These lines carry EBV–DNA and EBNA [4]. They have the same HL–A antigens, membrane immunoglobulin, Fc, C3 and EBV receptors as their original EBV-negative progenitor line, although with some quantitative differences [5]. They differ markedly in their "capping" patterns [6], Con A agglutinability [7] and a variety of growth characteristics *in vitro* [1, 8]. The EBV-negative lines are very sensitive to saturation conditions. The EBV converted lines grow to a comparable cell density but instead of dying abruptly at this point, they can maintain a high concentration of living cells for a considerably longer period of time. The sensitivity of the negative lines to saturation conditions is not merely due to the exhaustion of serum factors, because addition of fresh serum had no compensatory effect.

As reported in another publication [8] the EBV negative Ramos and BJAB lines also differ from their EBV-converted derivatives in their serum dependence. The EBV carrying sublines could grow on much lower serum concentrations than their EBV-negative progenitors.

In the present work, we have made a first attempt towards the analysis of the serum factors involved.

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MATERIAL AND METHODS

Cell lines

Two EBV-negative lymphoma lines were tested, Ramos (also called Ra 1) [9] and BJAB [10], together with their EBV carrying sublines,

converted by the B958 or the P3HR1 virus strains *in vitro*, as indicated in Table 1. Cells were routinely maintained in stationary suspension cultures at 37°C, in an atmosphere of 5% CO_2 in air and 80–90% relative humidity. RPMI-1640 (Gibco) medium was used containing 10% fetal calf serum (FCS) unless otherwise mentioned, supplemented with penicillin, streptomycin and fungizone. Cells were incubated in Falcon 3012 flasks in 10 ml medium. In experiments with low serum concentration, the medium was supplemented with 10 mM N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid (Hepes). For the experiments with dialysed serum FCS was dialysed at least 3 times against a twenty fold excess of RPMI-1640. In some experiments, dialysed FCS was "reconstituted" with RPMI-1640 that has previously served as the dialysing environment in a proportion of 900:100 ml FCS. The final concentration of the added dialysate corresponded to the dialysable part of 10% FCS. Reconstitution of dialysed FCS was also performed adding an H_2O dialysate of FCS, concentrated by low pressure evaporation to the original volume of the dialysed FCS.

Cell counting

Cell number and percent viability were assessed with a Coulter Counter (Model B Coulter Electronics Inc.) and with trypan blue.

Colony formation

The cells were tested for colony forming ability in agarose, with a feeder layer. Human embryo lung fibroblasts (10^5) were seeded into microplate wells (Falcon 3040). When the fibroblasts covered the bottom of the well they were exposed to 6000 r (Siemens roentgen unit 220 kV, 15 mA, filtration 1 mm aluminium) and covered with 0.05 ml 0.45% agarose (Induboise A37, L'industrie Biologique Française S.A.) in RPMI-1640 with 20% FCS. Twenty-four hours later 0.1–0.2 ml of 0.35% agarose in RPMI-1640 with 20% FCS containing different cell numbers was layered on the top of the feeder layer. The cultures were allowed to solidify and incubated at 37°C, 5% CO_2 in air, in a high humidity box.

RESULTS

Figure 1 shows the growth of Ramos and 3 of its EBV converted sublines at different FCS concentrations. In order to keep the level of serum constituents relatively close to the initial concentration, two thirds of the medium were replaced with new medium every second day. The initial cell concentration was 0.7–1.1 $\times 10^5$/ml. Cells were sampled on day 2, 4 and 6 when the experiment was terminated. By that time the cells reached a relatively high density and it became difficult to maintain the serum component level, even by frequent change. As shown in Fig. 1, all 3 EBV converted lines grew on 0.6% serum or less whereas the EBV negative cells failed to grow on 0.6% FCS or less. Smaller but significant differences were seen in the growth of the individual converted lines at low serum concentrations. EHRB-Ramos did not grow in 0.3% RCS, compared to EHRA-Ramos and II-WA-Ramos which grew well. In another series of experiments we measured the growth of

Table 1

Cell designation	EBNA	Converted by*	No. of EBV genome copies/cell	Producer state†	Reference
Ramos (RA 1)	–	–	<1	–	[9, 4]
AW-Ramos	+	P	1	–	[9, 5, 4]
II-WA-Ramos	+	P	4	–	[9, 5]‡
EHRA-Ramos	+	P	9	–	[9, 5, 4]
EHRB-Ramos	+	P	(average 17)	+	[9, 5, 4]
Ramos/B958	+	B	N.T.§	–	[3]‖
Ramos/HRIK	+	P	N.T.	–	[3]
BJAB	–	–	<1	–	[10, 11]
BJAB/B958	+	B	N.T.	–	[3]
BJAB/HRIK	+	P	(average 40)	+	[3]‖

*B = EBV derived from the B958 cell line.
P = EBV derived from the P3HR1 cell line.
†Negative = No EA or VCA positive cells.
Positive = A small fraction (0.5–2%) of EA and VCA positive cells.
‡Maria Andersson, personal communication.
§Not tested.
‖Herald zur Hausen, personal communication.

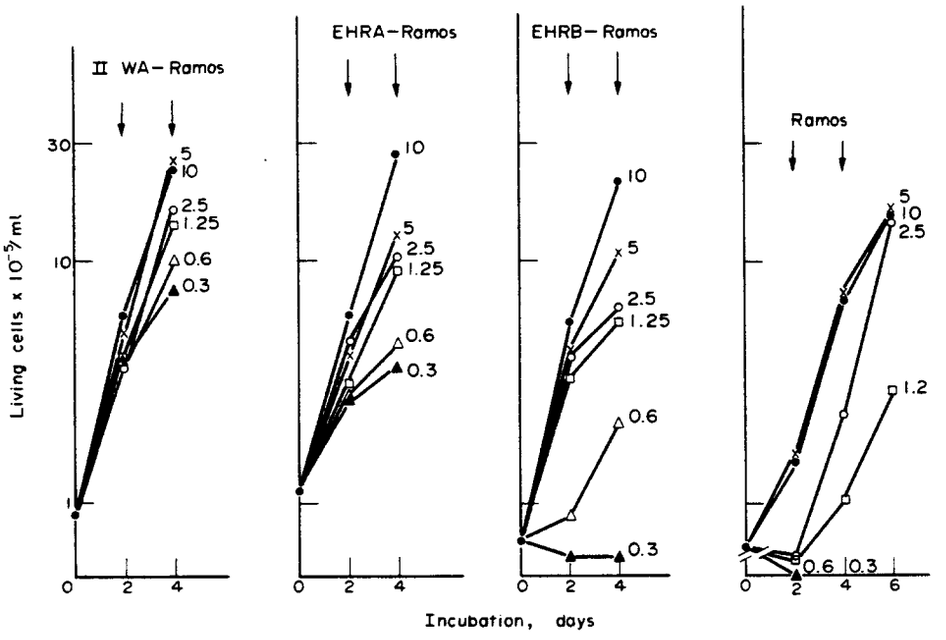

Fig. 1. Growth of Ramos and three EBV-converted Ramos sublines, II-WA-Ramos, EHRA- Ramos and EHRB-Ramos, in RPMI-1640 with 10 mM Hepes containing different concentrations of FCS. Cells were washed × 2 with PBS and then incubated in 10 ml medium at an initial concentration of 0.7–1.1 × 10^5 cells/ml. Two thirds of the medium were replaced with the corresponding fresh medium on day 2 and 4. Ramos did not grow on 0.6% FCS. The numbers to the right indicate percent FCS.

the EBV converted lines, in comparison with the original negative lines, after transfer to medium that contained serum concentrations ranging from 10 to 0.15%, but where the medium was not renewed during the experiment, in contrast to the previous series (Table 2). Growth was measured by daily cell counts and was approximately linear. All converted lines showed a decreased dependence on some serum factor(s) compared to the original Ramos and BJAB, like in the first experiment. A comparison between the two experimental designs shows that Ramos was capable of growing in 1.2% serum when the serum was regularly renewed (Fig. 1) but not in the absence of serum renewal (Table 2). Table 2 also shows that the cell numbers increased significantly in nearly all converted lines kept in 0.6 and 0.3% FCS. Ramos/B958 and Ramos/HR1K grew very well even at 0.15%. There was no systematic difference between the converted lines depending on the source of the converting virus.

Table 2. Growth of Ramos, BJAB and their EBV converted lines at different serum concentrations. The medium was not renewed during incubation

	Experiment*	Incubation period (days)	Living cells × 10^{-4}/ml					
			Initial serum concentration (%)					
			10	2.4	1.2	0.6	0.3	0.15
Ramos	I	4	120	33	0	0	0	0
BJAB	II	4	90	70	10	0	0	0
EHRA-Ramos	I	7	80	92	60	55	25	15
	III	6	230	N.D.†	160	65	25	11
EHRB-Ramos	I	7	134	60	52	2	0	0
	III	6	220	N.D.	80	30	7	3
Ramos/B958	II	4	205	N.D.	80	54	48	33
Ramos/HR1K	II	4	105	N.D.	86	52	55	70
BJAB/HR1K	II	4	82	N.D.	60	42	12	0

*The initial cell concentration was 15–25 × 10^4 cells/ml.
†Not done.

Dulbecco and Elkington [12] described a system in which the inhibition of growth *in vitro* caused by low serum concentration could be overcome by addition of Ca-ions. In our system the growth of the negative lines in low serum concentrations did not improve by adding Ca-ions to the medium (results not shown).

The differences between the EBV-negative and positive lines could be either due to a quantitative decrease in the requirement of the converted lines for some serum factor(s) needed by the original negative line and/or to the development of complete independence from some limiting serum factor(s).

As a first approach to this problem, cell growth was compared in 10% complete with 10% dialysed FCS. Figure 2 is one out of a series of experiments in which the two negative lines failed to grow on the dialysed serum altogether but grew on dialysed serum, reconstituted with the dialysate (see Material and Methods). All EBV converted sublines grew equally well on 10% normal and dialysed FCS.

The requirement of Ramos and BJAB for dialysable serum factor(s) was not absolute. It could be obviated by raising the cell density in the initial explant (Fig. 3). With initial cell concentrations of 1.6×10^4/ml or higher, both lines could "take off" on dialysed FCS.

Figure 4 shows an experiment where 10% dialysed FCS was reconstituted with different amounts of the concentrated dialysate. Both

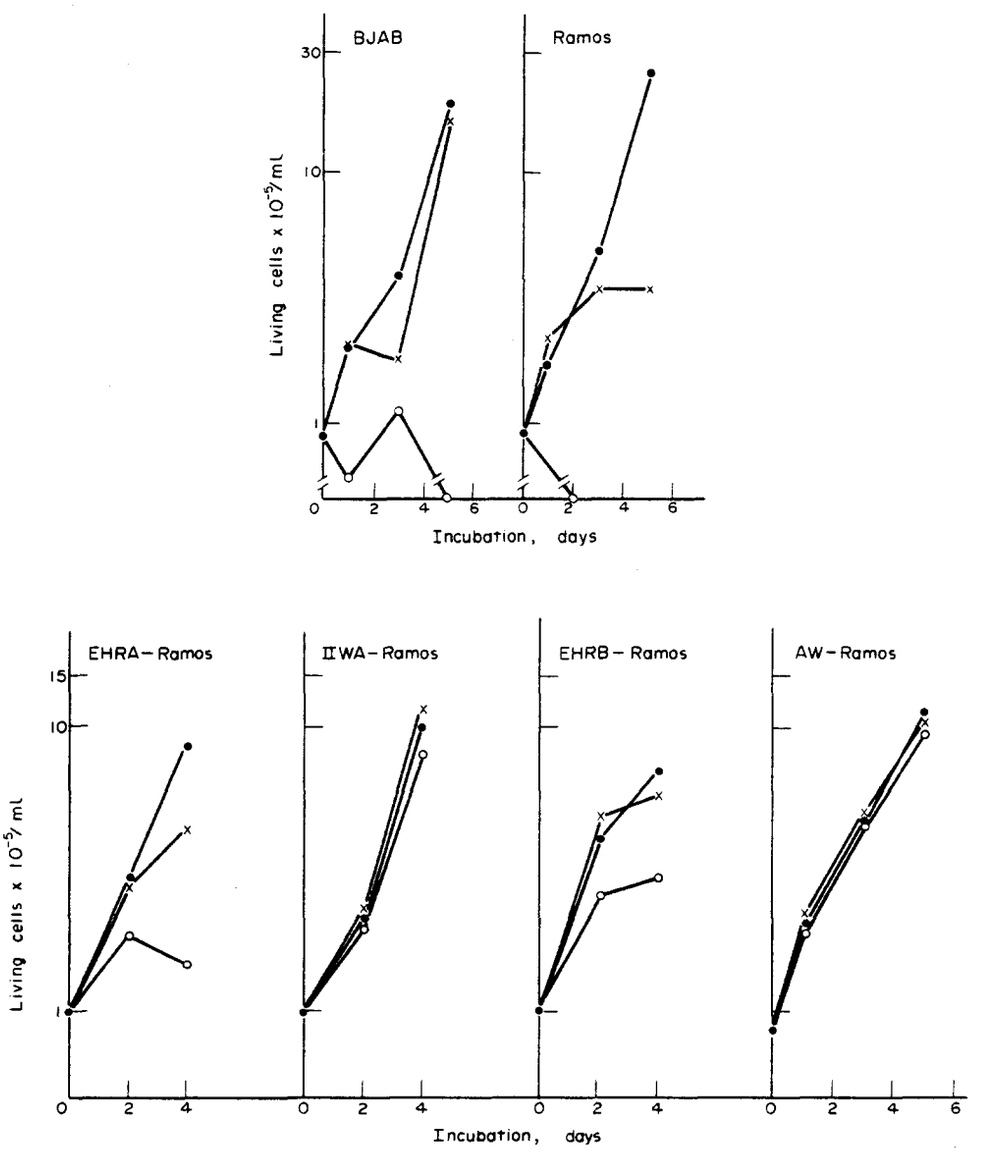

Fig. 2(A, B). Growth of Ramos, BJAB and four Ramos EBV-converted sublines in medium with 10% FCS (●——●), 10% dialysed FCS (○——○) and 10% dialysed FCS reconstituted with RPMI containing the FCS dialysate corresponding to 10% FCS (×——×).

negative lines could grow on reconstituted medium down to dialysate concentrations corresponding to 2% FCS. This is in good agreement with the serum dilution experiments presented above (compare Table 2 and Fig. 1). It is tempting to speculate that the limiting serum factors are at least partially identical in both experiments: if so, they would have to be dialysable.

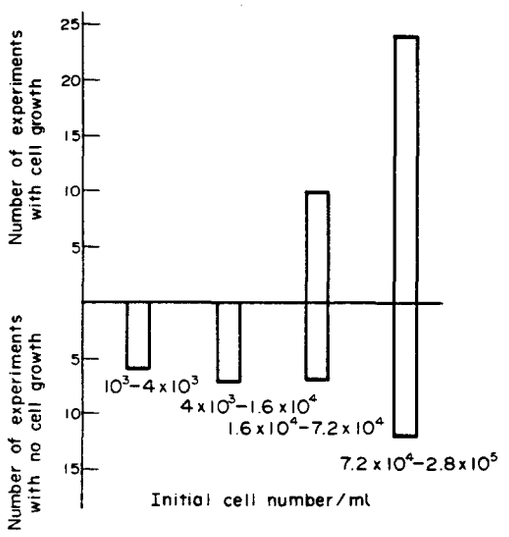

Fig. 3. The growth of Ramos and BJAB in 10% dialysed FCS in cultures inoculated with different numbers of cells. The control for each experiment shown was Ramos and BJAB cultures with 10% FCS with the corresponding cell number. All control cultures grew well. In parallel to many of the experiments exhibited, also some EBV positive cells were cultured in 10% dialysed FCS. In all these cases the cells grew well.

The cell lines were also compared for their ability to form colonies in soft agar. The negative lines did not form colonies in agarose on a human embryo lung fibroblast feeder layer. The EBV converted lines produced colonies but with different efficiencies (Table 3).

DISCUSSION

The present study confirms our previous finding [1, 8]. Conversion of two EBV negative human lymphoma lines, Ramos and BJAB leads to a decreased serum requirement. We have also shown that the negative lines are capable of growing at or slightly below the limiting serum concentration provided the serum is continuously renewed. The positive lines could grow at lower serum concentrations and even in the absence of medium renewal.

In the present paper, we also found that the two EBV negative lines differed from their converted sublines with regard to their ability to grow on dialysed 10% fetal calf serum. While the negative lines failed to grow altogether, the converted lines grew readily. The negative lines

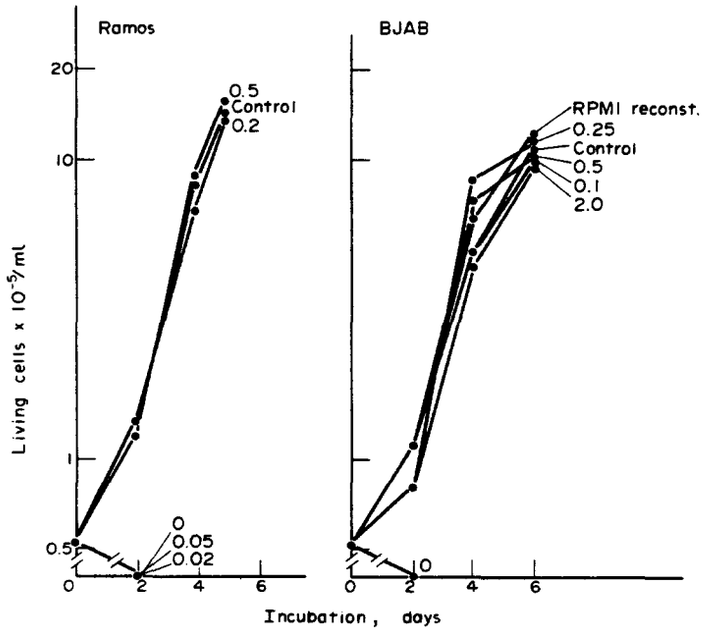

Fig. 4. Growth of Ramos and BJAB in medium with dialysed FCS and in reconstituted medium when 10% dialysed FCS was supplemented with different amounts of concentrated FCS dialysate. One ml concentrated dialysate was the dialysable equivalent of one ml serum. The numbers to the right indicate the volume of the concentrated dialysate (in ml) added per flask. "RPMI reconst." indicate the fully reconstituted medium.

Table 3. Colony formation in agarose. Cells were seeded into microplate wells containing human embryo lung fibroblasts as feeder layer. The number of colonies containing > 50 cells was scored after 8–10 days. Most of the colonies consisted of more than 100 cells

Cell line	No. of colonies			
	Initial no. of cells seeded per well			
	10^3	10^4	2×10^4	10^5
BJAB	0	0	0	0
BJAB/B958	N.D.*	120	250	N.D.
BJAB/HRIK	10	80	500	50
Ramos	0	0	0	0
EHRA-Ramos	0	60	140	N.D.

*N.D. = not done.

were able to grow on "reconstituted" medium, consisting of dialysed serum and the reintroduced, concentrated dialysate.

To some extent, the inability of the negative lines to grow on dialysed serum could be overcome by increasing the size of the inoculum.

In all likelihood, these changes were brought about by the EBV-conversion. The EBV positive sublines were selected on different occasions, by teams of investigators at two laboratories [3, 9]. Moreover, the same changes were found after EBV-conversion of two unrelated EBV negative lines. All this decreases the likelihood that the differences could have been due to some accidental event in connection with the selection procedure, rather than conversion to a viral genome positive status as such. The EBV associated changes are reminiscent of the decreased serum requirements characteristic for virus transformed monolayer cultures, derived from e.g. 3T3 or BHK-cells. The phenomenon was demonstrated after transformation with both DNA viruses (e.g. polyoma or SV40) and RNA viruses (e.g. RSV) [13, 14]. Like in the present case, the original line was already "immortalized" and aneuploid, and was changed further by the viral transformation. Conceivably, viral transformation may decrease the dependence of the cells on some serum factor(s). Holley [15] suggested that transformation leads to a membrane change that alters the interaction of necessary growth factors with membrane constituents. He visualized a change in the affinity or quality of relevant membrane receptors. Using partly the same EBV negative and converted lines as in the present study, Yefenof and Klein [6] previously showed that EBV conversion has led to certain changes in membrane behavior. This was expressed in a reduced ability of surface IgM and other membrane receptors to move in their lateral plane, as reflected in a reduced rapidity to redistribute and "cap" after contact with the appropriate ligand.

The differences between the EBV negative and positive lines with regard to their ability to grow in dialysed FCS suggested that the negative lines are dependent on some low molecular serum constituents. The recent demonstration [16] that small quantities of certain hormones may obviate the serum dependence of several lines warrants a search in that direction.

A further possibly EBV related difference between EBV negative and converted positive lines was restricted by the inability of the former to form colonies in soft agar, in contrast to the latter. This suggests a decreased dependence of the positive lines on some cell derived factors, capable to "condition" the medium.

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Radioimmunoassay for Epstein–Barr Virus (EBV)-associated Nuclear Antigen (EBNA). Binding of Iodinated Antibodies to Antigen Immobilized in Polyacrylamide Gel*

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Abstract—*A solid-phase radioimmunoassay was developed for the EBV-associated nuclear antigen (EBNA). Total homogenates of EBV-DNA and EBNA positive or negative cells were polymerized in polyacrylamide gel and compared for their ability to bind ^{125}I–IgG prepared from anti-EBNA positive and anti-EBNA negative sera. EBNA specific binding was demonstrated and confirmed by serological and cellular specificity controls. The assay allows the quantitation of antigen or antibody even in the presence of detergents and is suitable for biochemical characterization of the antigen. Reciprocal blocking studies with extracts from different cell lines showed quantitative and qualitative differences. One part of the EBNA specificiti(es) present in the human Burkitt lymphoma derived lines RAJI, DAUDI and AW-RAMOS was lacking in B95-8, a marmoset line carrying EBV derived from a human infectious mononucleosis line. This result may reflect differences in the viral genomes derived from Burkitt lymphoma and infectious mononucleosis lines or differences in the host cells.*

INTRODUCTION

B LYMPHOCYTES of human or simian origin can be transformed ("immortalized") by Epstein–Barr virus (EBV). The EBV genome present in these transformed cells convey on them the ability to grow and proliferate indefinitely *in vitro* [1, 2]. Under certain conditions EBV genome negative B lymphoma lines can be established as well [3, 4]. All EBV carrying cells, irrespective of their virus producer or nonproducer status, are characterized by the presence of EBV-associated nuclear antigen (EBNA, [5]). This antigen is of special interest since it may play a role in the maintenance of the immortalized state and/or the nonproducer status of the virus-DNA carrying, proliferating cell.

Several assays have been developed for the detection of a complement-fixing antigen in EBV transformed lymphoblastoid cells. An EBV-determined soluble complement-fixing antigen (CF-S) has been demonstrated in cell free extracts prepared from EBV genome carrying nonproducer cell lines [6–9]. Subsequently, a nuclear antigen (EBNA) was detected by anti-complementary immunofluorescence in fixed cells of EBV carrying lymphoblastoid lines and Burkitt lymphoma and nasopharyngeal carcinoma biopsy cells [5, 10–12]. There is suggestive evidence that CF-S and EBNA are closely related if not identical [13–15].

Previously a radioimmunometric assay was developed for EBNA, based on the direct and indirect binding of ^{125}I–IgG from polyvalent anti-EBV positive human sera, to nuclei prepared from nonproducer, EBNA positive cells or frozen–thawed cells [16, 17]. A certain quantitation of antigen and antibody became possible. Isolated nuclei or frozen–thawed cells as antigenic targets preclude the use of detergents or high salt concentrations in blocking assays aiming at antigen quantification. This is a serious limitation in experiments that require efficient antigen extraction and solubilization.

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This paper describes a radioimmunoassay for EBNA based on the immobilization of EBNA positive cells in a polyacrylamide gel matrix, EBNA is then quantitated by reaction with ^{125}I–IgG prepared from a polyvalent anti-EBV positive human serum.

MATERIAL AND METHODS

Chemicals

Carrier free Na^{125}I was obtained from the Radiochemical Centre Amersham, Bucks., England. Acrylamide, N,N'-methylene-bis-acrylamide (BIS) and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Kodak Co., Rochester, U.S.A.

Cell lines

The cell lines (Table 1; [3, 4, 18–23]) were grown in RPMI 1640 medium supplemented with 100 μg/ml streptomycin, 100 i.u./ml penicillin, 5 μg/ml fungizone and 10% heat inactivated foetal bovine serum.

Table 1. Properties of the cell lines used in this study

Cell line	EBV genome	EBNA	EA	VCA	Producer status	Reference
Ramos	–	–	–	–	–	[3]
BJAB	–	–	–	–	–	[4]
MOLT 4	–	–	–	–	–	[18]
AW-Ramos	+	+	–	–	–	[3]
NAMALWA	+	+	–	–	–	[19]
RAJI	+	+	+/–*	–	+/–*	[20, 21]
DAUDI	+	+	+	+	+	[19]
B95-8	+	+	+	+	+	[22]

*Low levels of EA positive cells (<0.1%) are found in RAJI cultures by autoradiography with iodine-labelled IgG from anti-EA positive sera [23].

Preparation of ^{125}I-labelled IgG

IgG was prepared from human sera by ammonium sulphate fractionation and chromatography on DEAE–sephadex A 50. Labelling of 50 μg of IgG with 1 mCi carrier free ^{125}Iodine was carried out using the chloramine T method described by Hunter and Greenwood [24]. The concentration of the labelled IgG was 10 μg/ml, the specific radioactivity was about 1×10^4 counts/min per ng protein. The labelled IgG was stored at 4°C in 0.02 M phosphate pH 7.2, 0.2% bovine serum albumin and 0.01% sodium azide.

Extraction procedure

Cells were harvested by centrifugation at 4°C, washed, resuspended in 0.02 M Tris/HCl pH 7.6, 0.15 M NaCl, 1.5 mM $MgCl_2$ at approximately 10^8 cells per ml and stored frozen at −79°C. The standard extraction procedure included three cycles of freezing and thawing (−79°/+37°C), homogenization with a Dounce homogenizer (9 strokes with a tight fitting pestle) and sonication in a MSE ultrasonic power unit at maximum power setting (1.5 A) for four 10-sec intervals. This homogenate was used for the preparation of immobilized antigen by polymerization in polyacrylamide gel. Alternatively, the homogenate was treated for 30 min at 4°C with 1% NP 40 (Nonidet P 40) prior to polymerization. In blocking assays with antigen containing extracts, the 100,000 × *g* (1 hr) supernatant fractions were used.

Preparation of immobilized antigen

Insoluble antigen was prepared by photopolymerization of cell homogenates within polyacrylamide gel using a modification of the method described by Carrel and Barandun [25]. Homogenate (0.8 ml) was mixed with 100 μl calf serum, 100 μl riboflavin (3 mg/ml), 5 ml 7.5% acrylamide containing 2.5% BIS and 2μl TEMED. After 1 hr at room temperature, the gel was homogenized with a loose fitting Dounce homogenizer and washed with 0.05 M Tris/HCl pH 7.5, 0.15 M NaCl until the supernatant became clear and the OD 280 was less than 0.05. In the case of NP 40 treated homogenates, the homogenized gel was washed with the same buffer containing 0.5% NP 40. More than 90% of the protein was incorporated in the gel matrix as determined by the Lowry method [26]. The gel suspension was stored at 4°C in the presence of 0.1% sodium azide.

Radioimmunoassay for EBNA

The homogenized gel was used as antigen

containing target material in a radioimmunoassay. In direct binding tests, 0.2 ml of packed gel suspended in 1 ml 0.05 M Tris/HCl pH 7.4, 0.15 M NaCl supplemented with 50% heat inactivated calf serum were incubated with ^{125}I–IgG (10–50 ng) prepared from a polyvalent anti-EBV positive serum "Adala" and an anti-EBV negative serum "BA". The mixtures were rotated overnight in the cold. Radioactivity bound to the gel was determined by crystal scintillation gamma counting after 4 washings with 0.05 M Tris/HCl pH 7.4, 0.15 M NaCl containing 20% calf serum. Blocking tests were performed either by preincubating the gel with various sera of known anti-EBV titers (Table 2; [27–31]) for 8 hr before adding ^{125}I–IgG "Adala" or by preincubating the iodinated IgG with 100,000 × ***g*** supernatant fractions from the cell homogenates of EBNA positive or negative cells for 8 hr prior to the addition of 1 ml gel suspension. Specific binding was defined as the difference between the binding of ^{125}I–IgG "Adala" and ^{125}I–IgG "BA". Blocking indices were calculated as follows:

$$BI = \frac{\text{specific binding from IgG "Adala"} - \text{specific binding from IgG "Adala" after preincubation with serum or extract}}{\text{specific binding from IgG "Adala"}}$$

Table 2. EBV antibody titers of human sera

Name	KCC No.	Diagnosis	anti-VCA	anti-EA		anti-EBNA	anti-MA (BI)
				D	R		
B.P.		HP	160	<10	<10	40	N.T.
L.H.		HP	160	<10	<10	20	N.T.
K.S.		HP	80	<10	<10	40	N.T.
B.S.		HP	80	<10	<10	80	N.T.
S.C.		HP	160	<10	<10	40	N.T.
L.W.		HP	160	<10	<10	80	N.T.
K.G.		HP	320	<10	10	40	N.T.
J.L.		HP	160	<10	10	80	N.T.
E.O.	1743	NPC	1280	320	N.T.	80	0.68
M.B.	1154	BL	320	10	80	20	0.65
N.N.	1629	BL	640	40	10	20	0.96
R.S.	1664	BL	320	<10	10	40	1.00
N.J.	976	BL	80	<10	320	160	0.68
A.O.	812	BL	1280	40	640	160	0.95
A.M. (Adala)	818	BL	2560	320	1280	80	0.95
M.A.	1530	BL	1280	10	40	80	N.T.
−1.		IM	320	40	N.T.	<2	N.T.
−2.		IM	160	20	N.T.	<2	N.T.
−3.		IM	160	40	N.T.	<2	N.T.
−4.		IM	640	<10	N.T.	<2	N.T.
−5.		IM	540	160	N.T.	<2	N.T.
−6.		IM	540	80	N.T.	<2	N.T.
−7.		IM	320	40	N.T.	<2	N.T.
−8.		IM	320	10	N.T.	<2	N.T.
−9.		IM	640	20	N.T.	<2	N.T.
−10.		IM	640	20	N.T.	<2	N.T.
E.K.		NC	<5	<5	<5	<2	0.06
R.K.		NC	<5	<5	<5	<2	0.00
BA		NC	<5	<5	<5	<2	0.00

KCC No. = Kenya Cancer Centre number of serum donor.
BL = Burkitt lymphoma.
NPC = Nasopharyngeal carcinoma.
HP = Healthy person, anti-EBV positive.
NC = Anti-EBV negative control.
VCA = Viral capsid antigen.
EA = Early antigen, D and R = subcomponents [27].
EBNA = EBV-associated nuclear antigen.
MA = Membrane antigen, BI = blocking index against FITC-Mutua [28, 29].
N.T. = Not tested.

The anti-VCA, anti-EA and anti-EBNA titers of the sera were determined by Henle and Henle, Philadelphia, in the course of previous collaborative studies (30, 31, Svedmyr, Jondal, Henle and Klein. To be published).

RESULTS

^{125}I–IgG binding to EBNA containing polyacrylamide gel (PAG)

Total homogenates of EBV-DNA and EBNA positive nonproducer cells (NAMALWA, AW-Ramos) and EBV negative cells (MOLT 4, Ramos) were photopolymerized in PAG. The homogenized gel was used in a direct binding assay with ^{125}I–IgG prepared from an anti-EBNA positive serum "Adala" and from a negative control serum "BA". Figure 1 illustrates a typical experiment. The gels containing EBNA positive cell homogenates showed specific

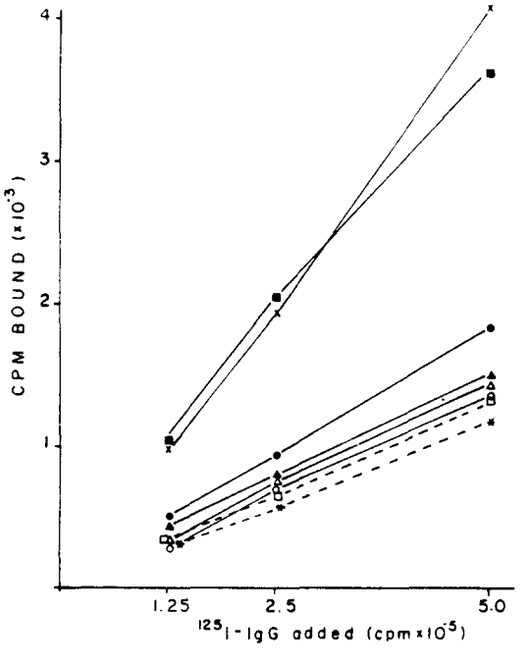


*Fig. 1. Binding of ^{125}I–IgG from anti-EBNA positive "Adala" and anti-EBNA negative "BA" serum to homogenized PAG containing homogenates of EBNA positive (AW-Ramos, Namalwa) and EBNA negative cells (Molt 4, Ramos), resp. Ramos (●—● Adala, ○—○ BA), Molt 4 (▲—▲ Adala, △—△ BA); Namalwa (■—■ Adala, □—□ BA), AW-Ramos (× —× Adala, *—* BA).*

binding of ^{125}I–IgG "Adala", in contrast to EBNA negative controls. The binding of ^{125}I–IgG "Adala" is apparently due to EBNA. detected even after cell homogenization and PAG-immobilization. The sensitivity of this assay permits EBNA to be determined with homogenates prepared from as few as $1-2\times10^6$ cells. The binding of iodinated IgG to EBNA is linear with increasing amounts of IgG added. This indicates that the antigen content of the homogenized gel is not limiting under the experimental conditions. This is of special importance for the interpretation of the results shown in Fig. 2. These data demonstrate the

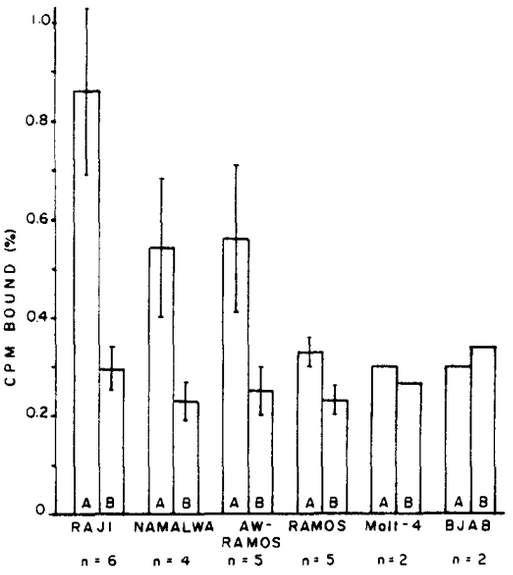


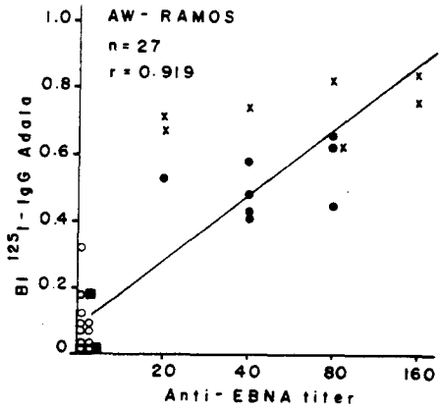
Fig. 2. Comparison of anti-EBNA positive ^{125}I–IgG "Adala" (A) and anti-EBNA negative ^{125}I–IgG "BA" (B) binding to EBV genome negative and positive cell homogenates polymerized in PAG. The standard deviations are indicated. Counts/min bound (%) were calculated from experiments as shown in Fig. 1.

EBNA specificity of the assay. The binding of iodinated IgG to PAG-homogenates prepared from NAMALWA or AW-Ramos was nearly identical, whereas the binding to RAJI-PAG was higher. The mean specificity ratios, i.e. counts/min bound from "Adala" divided by the counts/min bound from "BA", were 2.92 ± 0.44 (RAJI), 2.27 ± 0.44 (AW-Ramos) and 2.36 ±0.33 (NAMALWA). By using an autoradiographic method and ^{125}I–IgG of anti-EA positive sera, Moar *et al.* [23] recently showed that RAJI cells express EA in a low proportion ($<0.1\%$), whereas NAMALWA and AW-Ramos were completely negative for EA. It is conceivable that the assay described here detects both EBNA and low levels of EA in RAJI cells. It is also evident from Fig. 1 that the specific antibody concentration in the ^{125}I–IgG preparation is limiting under the conditions of the test. Therefore, an increased binding of ^{125}I–IgG "Adala" to RAJI-PAG compared to NAMALWA or AW-Ramos-PAG does not indicate a quantitative difference in a single antigenic specificity, but rather a qualitative difference, i.e. the existence of more antigenic specificities.

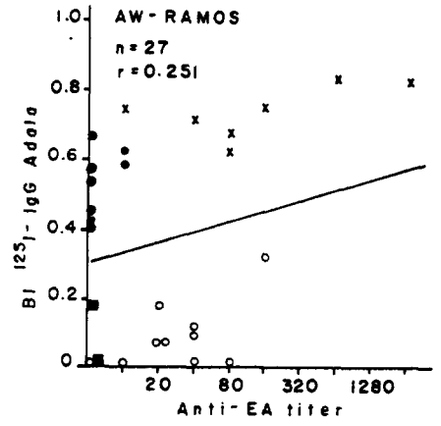
The 3 EBV-genome negative cells bound the EBV positive and negative reagents to nearly the same extent. The binding of ^{125}I–IgG "Adala" to the EBV genome negative PAG-homogenates was significantly lower than the binding to the corresponding EBNA positive target.

Blocking of ^{125}I–IgG binding by anti-EBV positive and negative sera

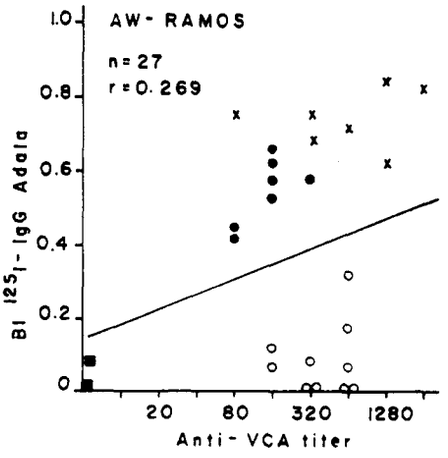
To check the EBNA specificity of the assay, blocking tests were carried out with a battery of sera with known anti-EBV antibody levels (Table 2). Figure 3 shows the blocking indices in the radioimmunoassay, plotted against the anti-EBNA, anti-EA and anti-VCA titers, respectively. There was a good correlation between the anti-EBNA titer and the blocking



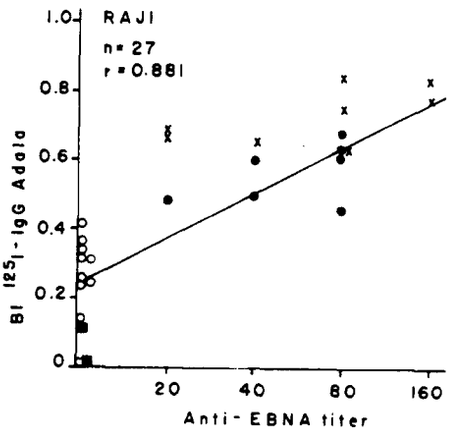
(A)



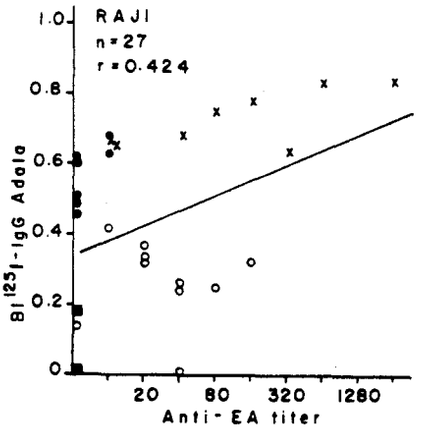
(B)



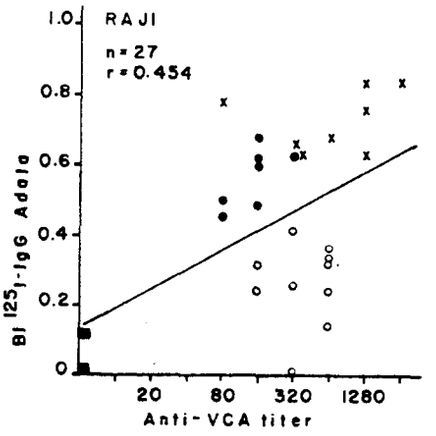
(C)



(D)



(E)



(F)

Fig. 3. Correlation between the anti-EBNA, anti-EA and anti-VCA titers of sera (10 µl undiluted serum) with known anti-EBV reactivity (Table 2) and their ability to block the radioimmunoassay. PAG-homogenates of AW-Ramos (A, B, C) and Raji cells (D, E, F) were used as targets. (●) anti-EBV positive sera of healthy donors; (×) Burkitt lymphoma or nasopharyngeal carcinoma patients; (○) early infectious mononucleosis patients; (■) anti-EBV negative sera.

activity with AW-Ramos and RAJI-PAG homogenates, whereas there was no significant correlation between blocking and the anti-EA or anti-VCA titers. Anti-EBNA positive sera of healthy donors and tumor patients blocked the binding of ^{125}I–IgG "Adala" to the AW-Ramos antigen preparation in direct relation to their anti-EBNA titers, whereas the anti-EBNA negative but anti-EA and -VCA positive sera of IM patients did not block or showed only a slight inhibition. Similar results were obtained with the RAJI preparation, with the exception that IM sera gave a partial blocking. This may be related to the elevated anti-EA antibodies in these sera, together with the presence of small amounts of EA in RAJI, but not in AW-Ramos cells. However, there was no overall correlation between the blocking and anti-EA or anti-VCA titers in the total material, also suggesting that EBNA is the dominating antigen detected in RAJI. In a control experiment (Fig. 4), the binding of ^{125}I–IgG "Adala" to Ramos homogenate immobilized in PAG was not significantly lowered by preincubation with the same sera.

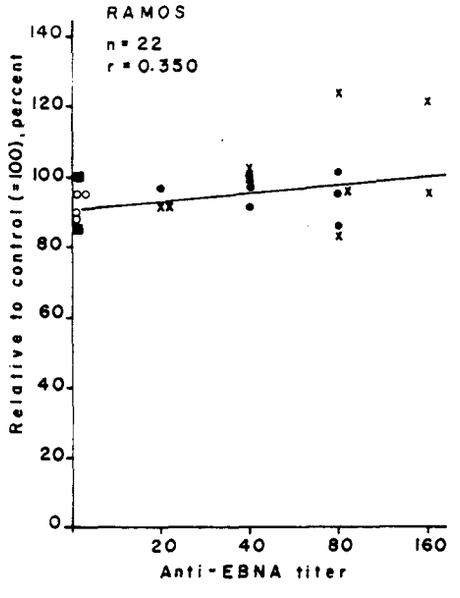


Fig. 4. Influence of preincubation of Ramos-PAG-homogenate with sera (10 µl undiluted serum) with different anti-EBNA titers on the binding of ^{125}I–IgG "Adala". The radioactivity bound to the gel is expressed as the percentage of a control incubation with ^{125}I–IgG "Adala" alone (= 100%). For symbols see Fig. 3.

Inhibition of ^{125}I–IgG binding by cell extracts

The specificity of the assay was also checked by inhibition tests with extracts from a variety of EBV genome carrying and negative lines. The test system offers the possibility to measure the amount of the relevant antigen even in the presence of detergents. Cross-inhibition tests may allow the dissection of qualitative differences. Figure 5 shows the data obtained in an experiment with 100,000 × *g* supernatants of NP 40 treated cell homogenates. RAJI and AW-Ramos extracts inhibited the binding of ^{125}I–IgG "Adala" to AW-Ramos PAG-homogenate nearly completely, whereas the EBNA negative BJAB extract had no effect. This suggests that AW-Ramos and RAJI contain identical or largely cross-reactive EBNA.

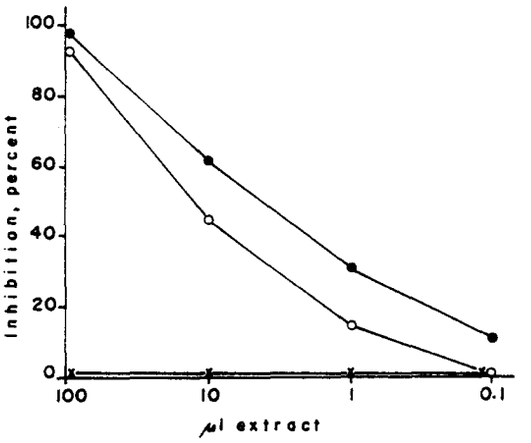


Fig. 5. Inhibition of ^{125}I–IgG "Adala" binding to AW-Ramos PAG-homogenate by preincubation of iodinated antibody with 100,000 × g supernatants of NP 40 treated total homogenates of BJAB (× — ×), AW-Ramos (●—●) and Raji cells (○—○).

Table 3 shows the results of an experiment with NP 40 treated (1% final concentration) total homogenates of 6 different cell lines. The iodinated antibody was preincubated for 8 hr with a cell homogenate prepared from 10^7 cells per test. After centrifugation for 15 min at 40,000 × *g* the supernatant was tested on the different PAG-homogenates indicated. The EBV-DNA, EBNA negative extracts showed no or only a slight inhibition of ^{125}I–IgG "Adala" binding in contrast to the EBNA positive cell extracts which gave a good inhibition. These data provide evidence that the EBNA specificity(ies) in these lines are largely cross-reactive.

Figure 6 shows a comparative titration of cell extracts tested in an inhibition assay with RAJI PAG-homogenate. The binding of ^{125}I–IgG "Adala" was inhibited to the same extent by RAJI and DAUDI extracts prepared without detergent, whereas B95-8 extracts gave only partial blocking. Since B95-8 cells contain EBNA, EA and VCA, as detected by immunofluorescence, whereas RAJI cells contain only EBNA and some EA, this finding suggests a qualitative difference in antigenic specificities, probably relating to the EBNA specificity that

Table 3. Inhibition of ^{125}I–IgG "Adala" binding to PAG-homogenates prepared from EBV-genome, EBNA positive cells by preincubation with NP40 treated homogenates of EBV genome positive and negative cells

Homogenates	PAG-homogenate prepared with AW-Ramos	Namalwa	Raji
Ramos	5	13	0
BJAB	17	18	7
Molt 4	6	3	0
AW-Ramos	94	70	70
Namalwa	89	85	63
Raji	75	86	93

Homogenates corresponding to 10×10^6 cells was used per test incubation. The data show the percent inhibition related to a control incubation with ^{125}I–IgG "Adala" alone.

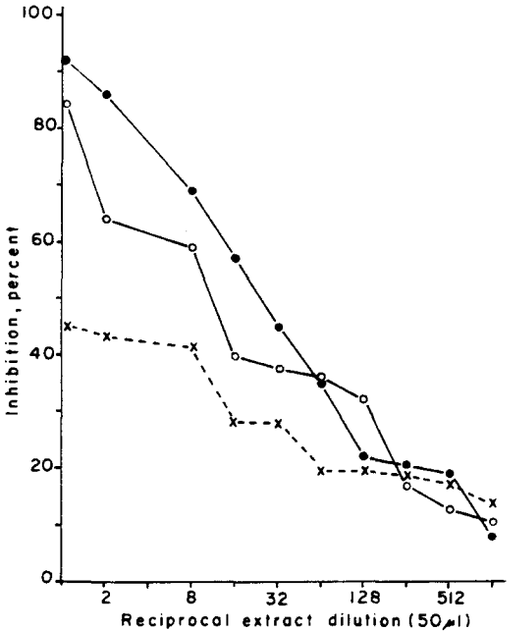


Fig. 6. Inhibition of ^{125}I–IgG "Adala" binding to Raji PAG-homogenate by preincubation of iodinated antibody with 100,000 × g supernatants of homogenates from Raji (○—○), Daudi (●—●) and B95-8 cells (×—×).

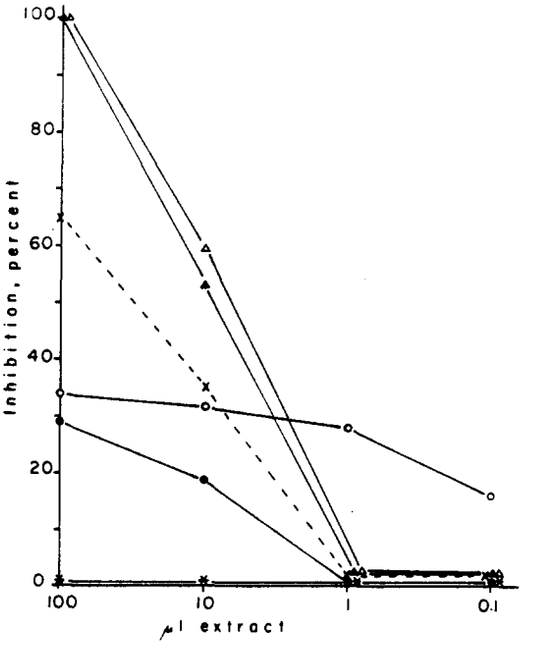


Fig. 7. Inhibition of ^{125}I–IgG "Adala" binding to Raji PAG-homogenate by preincubation of iodinated antibody with 100,000 × g supernatants prepared from NP-40 treated B95-8 (○—○), Raji (▲—▲), IUDR treated Raji (△—△), AW-Ramos (×—×), BJAB (—*) homogenates and a B95-8 extract (●—●), that was not exposed to NP40.*

dominates the test. Figure 7 shows a repeat test with additional cell extracts. Again, the detergent free B95-8 extract did not block the binding of ^{125}I–IgG "Adala" to the RAJI PAG completely. A corresponding extract prepared with NP 40 gave a slightly better, but still only partial blocking with a clear plateau. This suggests a qualitative difference. In contrast, the extracts of RAJI or IUDR treated RAJI (60 µl/ml medium for 2 days) blocked the binding of ^{125}I–IgG "Adala" completely at high concentrations, AW-Ramos extract showed less inhibition. This may have been a quantitative rather than a qualitative difference, since the curve had a similar shape and there was no plateau. Alternatively, the lack of EA in this cell line may have been responsible.

DISCUSSION

The method of protein immobilization in polyacrylamide gel introduced by Carrel and Barandun [25] was initially established for immunosorbent procedures. Subsequently, proteins fixed in polyacrylamide gel turned out to be useful as antigen sources in solid-phase radioimmunoassays for cell membrane antigens [32, 33]. In the present study, a radioimmunoassay for EBNA was developed based on this technique.

Total homogenates prepared from EBNA positive or negative cells polymerized in PAG served as the antigen. EBNA specificity could be confirmed by serum blocking and antigen inhibition tests. A collection of anti-EBV positive and negative sera was selected for the blocking tests, including positive sera that were discordant with regard to their anti-EBNA vs anti-EA or anti-EA and anti-VCA titers. Blocking of the radioimmunoassay showed a good correlation with the anti-EBNA titers, but not with the anti-EA or anti-VCA titers, provided that an EBNA positive nonproducer line was used for antigen preparation. However, if EA+, VCA+ producer cells were used as antigen sources, EA and VCA could be detected by this assay (Dölken and Klein, manuscript in preparation). EBNA positive nonproducer cells are therefore a prerequisite for monospecific EBNA detection.

Comparative binding studies with different EBV-genome positive and negative cells showed that the reference reagent "Adala" detects an EBV-associated specificity. Taking into account the immunofluorescence data and results obtained with autoradiography in case of AW-Ramos and NAMALWA (Table 1), EBNA was the only conceivable EBV-associated antigenic specificity. In RAJI cells, the specificity preferentially detected is EBNA, but possibly small amounts of EA are also detected.

Blocking assays with extracts from various EBNA positive producer and nonproducer lines were carried out to compare their EBNA content quantitatively and qualitatively. With RAJI PAG-homogenates as the antigen, the binding of ^{125}I–IgG "Adala" was completely blocked by RAJI, IUDR treated RAJI and DAUDI extracts. B95-8 cell extracts prepared with or without the detergents NP 40 did not inhibit completely (30–45% inhibition) and a plateau of inhibition was observed. The extract prepared from EA−, VCA−, MA−, EBNA+ AW-Ramos cells blocked more extensively and with no plateau effect compared to the EA+, VCA+, MA+, EBNA+B95-8 extract. In a separate experiment, the extracts of AW-Ramos and NAMALWA inhibited the binding of ^{125}I–IgG "Adala" to about the same extent, but still to a larger extent than the B95-8 extract. Taken together, these findings suggest that the EBNA of the B95-8 cells is partially different from the EBNA in AW-Ramos, NAMALWA, RAJI and DAUDI.

This finding appears particularly interesting in view of the origin of the different cell lines. RAJI, DAUDI, and NAMALWA are EBV carrying Burkitt lymphoma lines. AW-Ramos was derived by converting the EBV genome negative Ramos line with P3HR-1 virus, produced by the Burkitt lymphoma line P3HR-1 and is now an EBV-DNA, EBNA positive nonproducer line. The B95-8 line was established by infecting marmoset cells with EBV derived from the 833 L line of infectious mononucleosis (IM) origin. There is now accumulating evidence that the molecular weight of the circular DNA isolated from IM derived lines is about 12% smaller than the DNA obtained from Burkitt lymphoma lines (Adams *et al.*, manuscript in preparation). Also the findings of Pritchett *et al.* [34] showed that B95-8-EBV-DNA lacked approximately 15% of the DNA sequences of the P3HR-1-EBV-DNA. The possible difference in EBNA specificities between the B95-8 extract and the various Burkitt lymphoma lines tested may reflect the difference in the viral genomes. Alternatively, however, it is also possible that the difference described in this paper is related to the difference in the host cell species, human vs marmoset. Since both, the P3HR-1 and the B95-8 viruses have been now successfully introduced into the same EBV genome negative lines [35], this question can be subjected to critical examination.

In this paper data were presented showing that a test for EBNA can be established by using PAG immobilized homogenates of antigen positive cells. By using EBV genome positive producer cells that are positive for all known EBV-associated antigens, a radioimmunoassay detecting EBNA, EA and VCA has been established (Dölken and Klein, manuscript in preparation).

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Urinary Steroid Profiles in Normal Women and in Patients with Breast Cancer in Britain and Japan: Relation to Thyroid Function

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Abstract—*Steroid profiles of urinary 17-oxosteroids, pregnanediol and pregnanetriol were studied by gas–liquid chromatography in normal Japanese and British women, and in women with breast cancer from both races. The results showed that both pre- and post-menopausal Japanese women excreted significantly less aetiocholanolone, 11-oxo aetiocholanolone, and 11-hydroxy aetiocholanolone than their British counterparts. Androsterone was significantly less in Japanese pre-menopausal women and pregnanetriol in post-menopausal women respectively..*

However, a significantly lower androsterone (5α): aetiocholanolone (5β) ratio was found in the Japanese cancer patients than in the normal Japanese women (pre-menopausal, $P = < 0.02$, post-menopausal, $P = < 0.05$).

In the British pre-menopausal breast cancer patients this 5α:5β ratio was higher than in the normal British control ($P = < 0.02$).

A comparison of blood plasma TSH levels with the urinary androsterone: aetiocholanolone ratios gave a significantly negative correlation in both the Japanese normal and cancer cases ($P = < 0.05$). No agreement was found in a similar comparison in the British women.

These results could signify that a diminished thyroid function may be a predisposing factor to breast cancer in Japanese women.

INTRODUCTION

THE ROLE of the thyroid in the aetiology of breast cancer is still not clear. Some early reports indicated that hypothyroidism was more common in women with breast cancer than among the normal population [1–3]. More recently, Mittra and Hayward [4] have shown that plasma TSH (thyroid stimulating hormone) was significantly higher in patients with early or advanced breast cancer, than among women in hospital with illnesses unrelated to breast disease. Furthermore, Mittra, Hayward and McNeilly [5] have postulated that suboptimal levels of circulating thyroid hormones may abnormally sensitize mammary epithelial cells to prolactin stimulation, which may lead to eventual neoplasia. Certainly, once breast cancer has been diagnosed, patients with a history of hypothyroid disorders have a much more rapid recurrence rate after mastectomy, together with a poorer survival experience than euthyroid or hyperthyroid women [6]. The ratio of the urinary androgen metabolite androsterone 3α-OH-5α-androstan-17-one to its isomer aetiocholanolone 3α-OH-5β-androstan-17-one has been shown to be a particularly sensitive index of thyroid function. This index which has been termed the 5α:5β ratio is high (i.e. > 2.0) in hyperthyroidism and low in myxoedema (i.e. > 0.25) [7, 8]. It has been found that normal Japanese women have a significantly higher 5α:5β ratio than comparable British women [9, 10] and it has been suggested that the lower incidence of breast cancer in Japanese women, compared with British or American women [11], might be due to high thyroid activity in the Japanese [10].

Methods are now available for an extensive survey of urinary androgen metabolites and other steroids. Similarly, TSH assays are now possible. This paper describes a study of the

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relationship of these factors in normal Japanese and British women and patients with breast cancer.

MATERIAL AND METHODS

The normal Japanese women comprised 20 pre-menopausal and 15 post-menopausal healthy women living in Tokyo. The normal British group were 19 pre-menopausal and 15 post-menopausal healthy white women living either in Guernsey or the London area. The Japanese breast cancer cases (26 pre-menopausal and 17 post-menopausal women) were from the National Cancer Center Hospital, Tokyo. The British breast cancer cases of 11 pre-menopausal and 21 post-menopausal women were from Guy's Hospital, London. Both groups consisted of early and advanced cases. Three 24 hr collections of urine were made at home by the normal volunteers, whilst the 3 urine specimens from the patients were collected in hospital before operation. All the specimens were stored in plastic (PVC) containers at −20°C; those from the Japanese were flown to London in insulated crates.

The urine was processed and analysed by gas–liquid chromatography using the method of Bailey, Fenoughty and Chapman [12], except that the enzyme used for the hydrolytic cleavage of the steroid glucosiduronate conjugates was β-Glucuronidase Pasteur d'origine bacterienne [13]. The gas chromatograph was a Becker (model 420) and the glass capillary column (25m) coated with methyl silicone (OV101) stationary phase was obtained from LKB Ltd. The plasma TSH assays used for comparison with the urinary 5α : 5β ratios have already been published [14].

The urinary steroids measured were as follows:

Androsterone
(3α-hydroxy-5α-androstan-17-one;
Aetiocholanolone
(3α-hydroxy-5β-androstan-17-one);
11-Oxo-aetiocholanolone
(3α-hydroxy-5β-androstan-11, 17-dione);
11-Hydroxy-androsterone
(3α, 11β-dihydroxy-5α-androstan-17-one);
11-Hydroxy-aetiocholanolone
(3α, 11β-dihydroxy-5β-androstan-17-one);
Pregnanediol
(3α, 20α-dihydroxy-5β-pregnane);
Pregnanetriol
(3α, 17β, 20α-trihydroxy-5β-pregnane).

Assessment of method

The assessment of the method is presented in Table 1.

RESULTS

(a) Normal Japanese and British women

The urinary steroid excretion of normal pre- and post-menopausal Japanese and British women is shown in Table 2. The Japanese pre-menopausal women excreted significantly less androsterone, aetiocholanolone, 11-oxo aetiocholanolone and 11-hydroxy aetiocholanolone than the British women. The 5α:5β ratios of both androsterone : aetiocholanolone and 11-hydr-

Table 1. The standard deviation and coefficient of variation were calculated from replicate analyses on the same sample

Steroid	μg/24hr	S.D. ±	Coefficient of variation	n	Recoveries (n=6)
Androsterone	411	36.7	8.9	10	—
Aetiocholanolone	631	90.0	14.4	7	*75.3% (±1.2)
11-oxo Aetiocholanolone	522	58.8	11.2	7	*73.0% (±8.4)
11-OH Androsterone	308	34.4	11.2	10	—
11-OH Aetiocholanolone	236	46.6	19.6	10	—
Pregnanediol	150	22.4	15.0	10	—
Pregnanetriol	139	17.4	12.5	10	—
5α:5β					
Andro: Aetio	0.65	0.08	12%		—
11-OH Andro: 11-OH Aetio	1.33	0.20	15%	10	—

*10 μg of steroid added to urine prior to hydrolysis.

Table 2

Steroid	Normal British and Japanese women Japanese pre-menopausal	British pre-menopausal	Japanese post-menopausal	British post-menopausal
A	779 (±289)†	1211 (±671)	501 (±350)	755 (±440)
E	672 (±250)§	1450 (±835)	390 (±280)	1044 (±704)
11-KE	146 (±77)	364 (±211)	143 (±109)‡	322 (±117)
11-OHA	626 (±171)	811 (±411)	650 (±490)	528 (±232)
11-OHE	173 (±102)§	451 (±255)	172 (±125)‡	329 (±213)
PD	1693 (±1082)	2596 (±1805)	163 (±104)	230 (±120)
PT	745 (±557)	1221 (±818)	104 (±77)*	217 (±134)
5α:5β (A:E)	1.25 (±0.47)‡	0.87 (±0.24)	1.43 (±0.55)§	0.82 (±0.30)
5α:5β (OHA:OHE)	5.35 (±4.98)*	2.28 (±1.33)	6.63 (±7.56)§	1.83 (±0.89)

A = androsterone, E = aetiocholanolone.
11-KE = 11-oxo aetiocholanolone.
11-OHA = 11-hydroxy androsterone.
11-OHE = 11-hydroxy aetiocholanolne.
PD = pregnanediol, PT = pregnanetriol.
*indicates $P<0.05$
†indicates $P<0.02$
‡ indicates $P<0.01$
§ indicates $P<0.001$
} Calculated by Mann–Whitney ranking test.
Figures in brackets indicate standard deviation.

oxy androsterone : 11-hydroxy aetiocholanolone in the Japanese pre-menopausal women were significantly greater than in the corresponding British group. A similar difference was found between the Japanese and British post-menopausal group with the exception that the mean androsterone excretion in the Japanese was not significantly different from that of the British women.

(*b*) *Patients with breast cancer*

The urinary steroid excretion of pre- and post-menopausal Japanese and British women with breast cancer is shown in Table 3.

Table 3

Steroid	Patients with breast cancer Japanese pre-menopausal	British pre-menopausal	Japanese post-menopausal	British post-menopausal
A	698 (±372)¶	1126 (±760)	470 (±310)	561 (±505)
E	729 (±332)‖	1006 (±464)	382 (±175)	612 (±430)
11-KE	170 (±107)	289 (±228)	170 (±100)‖	344 (±241)
11-OHA	620 (±216)	775 (±372)	640 (±430)	695 (±508)
11-OHE	174 (±118)††	386 (±199)	173 (±177)**	334 (±152)
PD	1676 (±1140)	2145 (±1626)	159 (±100)	262 (±161)
PT	734 (±405)	954 (±771)	131 (±80)	221 (±157)
5α:5β (A:E)	0.86 (±0.23)‖	1.25 (±0.52)	1.13 (±0.37)	1.01 (±0.37)
5α:5β (OHA:OHE)	4.82 (±3.27)	2.76 (±2.58)	3.84 (±2.31)‖	2.27 (±1.89)

A = androsterone, E = aetiocholanolone.
11-KE = 11-oxo aetiocholanolone.
11-OHA = 11-hydroxy androsterone.
11-OHE = 11-hydroxy aetiocholanolone.
PD = pregnanediol, PT = pregnanetriol.
‖indicates $P<0.05$
¶indicates $P<0.02$
**indicates $P<0.01$
††indicates $P<0.001$
} Calculated by Mann Whitney ranking test.
Figures in brackets indicate standard deviation.

As in the normal controls, the Japanese patients with breast cancer excrete less androsterone and aetiocholanolone than comparable British patients. The difference is significant in the pre-menopausal women but not in the post-menopausal. Similarly, 11-OHE excretion is significantly lower in Japanese patients than in British patients and 11-KE excretion was also lower both in Japanese controls and patients. Japanese pre-menopausal patients have a significantly lower 5α:5β ratio than British patients.

(c) Comparison of steroid excretion of normal women and patients with breast cancer within populations

When the results given in Tables 2 and 3 were compared, no significant differences emerged between normal Japanese women and Japanese women with breast cancer, except that the androsterone : aetiocholanolone ratios were lower in the cancer cases than in the controls (pre-menopausal, $P \leqq = 0.02$; post-menopausal, $P = < 0.05$). In a similar comparison in British pre-menopausal women the androsterone : aetiocholanolone ratio was significantly higher in the patients with breast cancer ($P < 0.02$). No significant differences were found in the post-menopausal women with the exception that the amounts of aetiocholanolone were lower than in the normal controls ($P = < 0.01$).

(d) Relationship between blood plasma TSH and urinary androsterone : aetiocholanolone ratio

The comparison between blood plasma TSH and the urinary androsterone : aetiocholanolone ratio in the Japanese women is shown in Fig. 1. There is an inverse correlation between the two sets of data that is significant ($r = -0.29$, $P = < 0.05$). A similar comparison of plasma TSH and the urinary androsterone : aetiocholanolone ratios in the British women in Fig. 2, shows no correlation whatsoever.

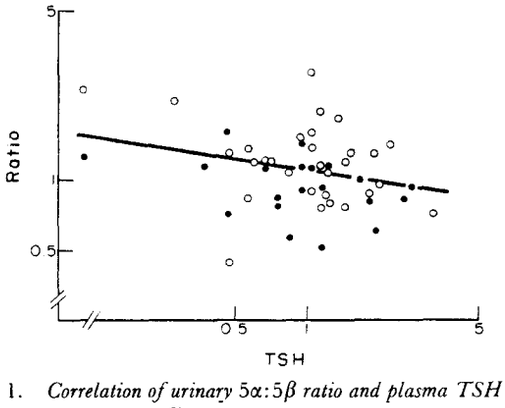

Fig. 1. Correlation of urinary 5α:5β ratio and plasma TSH in Japanese women.
○ = *Normal Japanese women.*
● = *Japanese women with breast cancer.*
$y = -0.21x + 1.25$ ($r = -0.32$, $P < 0.05$).

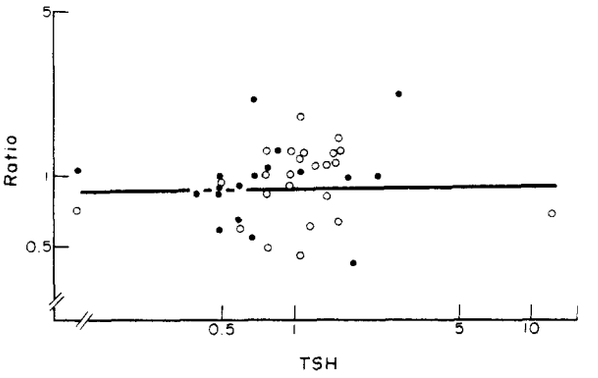

Fig. 2. Correlation of urinary 5α:5β ratio and plasma TSH in British women.
○ = *Normal British women.*
● = *British women with breast cancer.*
$y = 0.06x + 0.93$ ($r = 0.11$. *P—not significant*).

DISCUSSION

The results confirm earlier findings [9, 10] that the amounts of androsterone and aetiocholanolone excreted by normal Japanese women are lower than in British women, but the 5α:5β ratio of these two androgen metabolites is higher in Japanese women. Two urinary corticosteroid metabolites, 11-oxo aetiocholanolone and 11-hydroxy aetiocholanolone are excreted in smaller amounts by Japanese women, whilst the amounts of 11-hydroxy androsterone, a metabolite of 11-hydroxy-androst-4-en-3, 17-dione, are the same in both races. Thus, there should be a considerable difference in the activities of the 5β-reductase enzymes in the Japanese women to account for the relatively low aetiocholanolone, 11-oxo aetiocholanolone and 11-hydroxy aetiocholanolone. The latter two compounds are mainly derived from cortisol.

The steroid assays indicate a greater degree of thyroid activity in normal Japanese women in that the 5α:5β ratios of androsterone and aetiocholanolone, and of their 11-hydroxylated equivalents are higher than in the British women. Although the plasma TSH levels show no significant difference between the two races this may be due to these assays or the level of this hormone being less sensitive than the urinary steroids as an indicator of small changes in thyroid function. It is also possible that there may be differences in triiodothyronine (T_3) and thyroxine (T_4) levels in the two races. That the 5α:5β ratios are reliable indicators of major thyroid disturbances is beyond doubt [7, 8]. It is therefore reasonable to assume that smaller changes in thyroid metabolism are reflected by concomitant urinary steroid changes. The incidence of breast cancer may well be related to circulating levels of thyroid hormones and

enhanced thyroid activity could "protect" the Japanese population.

Another interesting feature of these results is that the urinary excretion of pregnanediol and pregnanetriol in pre-menopausal women is similar in both races. Only in the British post-menopausal group is pregnanetriol marginally higher. From this it may be deduced that the production rates and metabolic fate of progesterone and 17-hydroxy-progesterone are almost identical in both races. There are several differences between normal women and patients with breast cancer in each population. Aetiocholanolone levels are appreciably lower in the British cancer cases than in their normal controls thus confirming earlier findings [15, 16]. There was no difference between the similar group in the Japanese population. However, the comparison between normal Japanese women and those with breast cancer shows a difference in the androsterone : aetiocholanolone relationship in that the $5\alpha:5\beta$ ratio is significantly lower in the cancer cases resembling that of the normal British women. These differences do not occur among the normal British women and those with breast cancer, and in fact the $5\alpha:5\beta$ is actually significantly higher in the pre-menopausal cases. It is therefore possible that low thyroid activity could be correlated with increased risk of the disease in Japan. This possibility is further reinforced by the significant inverse correlation found between the androsterone : aetiocholanolone ratio and the blood plasma TSH values in the Japanese women. This feature is conspicuously absent among the British women.

Another piece of evidence suggestive of relatively high thyroid activity in normal Japanese women is that the plasma androstenedione level is lower than in British women [17]. It has previously been demonstrated that a diminished testosterone clearance rate together with a decrease in its conversion to androstenedione is present in hyperthyroidism [18].

Studies based on the rise of plasma TSH following intravenous injection of thyrotropin-releasing hormone (TRH) suggest a lower level of thyroid activity in British women with breast cancer than in women without the disease [4]. Therefore, on the basis of the results presented in this paper the TRH test should show a greater difference in TSH stimulation in Japanese women with breast cancer than among their control group and, in fact, this has been reported [19]. A relative degree of "hypothyroidism" appears to be present in Japanese women with breast cancer and this could be a factor in the aetiology of the disease in Japan. It must be emphasized that this conclusion is based on small and possibly unrepresentative samples from the British and Japanese populations and that changes now taking place in incidence rates, dietary and social habits may mean that subsequent studies would afford different results. But the results of this pilot study encourage us to pursue the present lead in larger cross sections of the two races.

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ICRF-159: Current Status and Clinical Prospects*

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Abstract—*The human clinical pharmacology, toxicity and antitumor activity of ICRF-159 are reviewed. Phase* II *trials, multi-drug combinations and adjuvant trials are discussed in reference to the future study of this agent.*

INTRODUCTION

MANY enzyme systems contain trace metals or depend upon them for activity. Furthermore, various chelating agents have been shown to affect the growth of cells and tumors [1]. Ethylenediamine tetraacetic acid (EDTA), one of the most potent chelating agents for divalent cations, has no significant antitumor activity [2]. This lack of activity has been ascribed to the high polarity of the molecule which would prevent entry into cells [3]. Less polar derivatives of EDTA have thus been synthesized with this approach culminating in the development of ICRF-159 [4].

A review of this new drug, with special emphasis on preclinical data, has been published recently [5]. The following analysis attempts to summarize the current clinical status of ICRF-159 and will hopefully define possible leads for further investigations.

EXPERIMENTAL DATA

ICRF-159 [(±), 1,2 bis(3, 5-dioxopiperazin-1-yl) propane, Razoxane or NSC-129943] is a bisdiketopiperazine derivative (Fig. 1) which was originally synthesized by the Imperial Cancer Research Fund Facilities in London, England. Its mechanism of action has not yet been fully elucidated. Using phytohemagglutinin stimulated human lymphocytes in synchronized cell culture, the late prophase and early metaphase (G_2-M) were found to be most sensitive to ICRF-159 [6]. In mouse fibroblast cell cultures, the drug inhibited DNA synthesis with little effect on RNA and protein synthesis, following a pattern reminiscent of the activity X-irradiation and radiomimetic agents [7]. In animal experiments, ICRF-159 was shown to inhibit the formation of platelet thrombi in blood vessels. This effect could not be related to a decrease in platelet counts or to a decrease in ionized serum calcium concentrations [8].

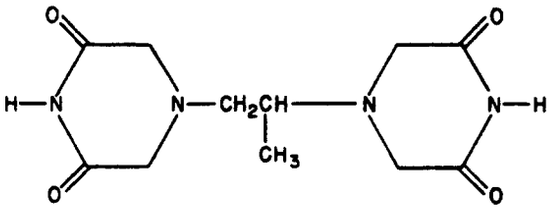

Fig. 1. *Structural formula of ICRF-159.*

Experimental antitumor effect was found in a wide range of animal tumors [4]. In the ascitic L1210 system, the drug exhibited schedule-dependency in accordance with its cell-cycle-phase-specificity, the optimal schedule being administration I.P. or P.O., every three hours on days 1, 5, 9.§ Like 5-fluorouracil and epipodophyllotoxin derivatives, ICRF-159 was shown to inhibit pulmonary metastases in the Lewis lung carcinoma at doses having little effect on the rate of growth of the primary implant [9, 10]. This inhibition was tentatively related to profound changes in the vascularization of the primary tumor [11, 12]. Antitumor synergism of ICRF-159 plus X-irradiation has been found in

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the sarcoma S-180 system [13]. Synergism was also reported with other cytotoxic agents including daunomycin, adriamycin, 5-fluorouracil and *cis*-diamminedichloroplatinum in the L1210 model and cyclophosphamide in the Lewis lung carcinoma [14–16]. Finally, it must be noted that ICRF-159 was found to reduce the cardiotoxic effects induced by daunomycin and adriamycin in the isolated dog heart [17].

CLINICAL PHARMACOLOGY

Following oral ingestion of ^{14}C-ICRF-159, fecal and urinary recovery of radioactivity appeared to depend upon the schedule of drug administration [18]. After a single dose of 3 g/m^2, the urinary recovery within 96 hr averaged only 23% (range, 13–34%). When the drug was given at 3 g/m^2 in 3 divided doses in one day, the urinary recovery increased to 52% (range, 42–65%) of the administered radioactivity. At each schedule, radioactivity unrecovered in the urine was largely accounted for in the feces. The bulk of the fecal radioactivity was chromatographically identical to ICRF-159 whereas all of the urinary radioactivity appeared to be metabolite.

TOXIC EFFECTS

Toxic effects encountered following ICRF-159 administration mainly include myelosuppression, gastrointestinal intolerance and alopecia (Table 1) [19–21].

Table 1. *Toxic effects of ICRF-159 in 80 patients**

Toxic effects	Number of patients with toxic effects
Hematologic	
WBC < 2000/mm^3	34
Platelets < 100.000/mm^3	17
Gastrointestinal	
Nausea	27
Vomiting	17
Diarrhea	3
Alopecia	36
Miscellaneous	
Flu-like syndrome	2
Dermatitis	1
Stomatitis	1

*Cumulative results of 3 trials with oral ICRF-159 1 g/m^2/day in 3 divided doses each day for 3 consecutive days [19–21].

Myelosuppression

Leukopenia is the dose-limiting toxicity induced by ICRF-159, as observed with the drug given 3 times daily for 1 or 3 days at a total dose of 3 g/m^2 per course [22, 23]. When the drug is administered as single doses up to 10.5 g/m^2, only mild and inconsistent myelosuppression is noted, probably reflecting the peculiar intestinal absorption of the drug [23]. At the daily times 3 schedule, leukopenia persists for 1 week with the nadir occurring at day 12 (mean) and complete recovery by day 21. On subsequent courses, leukopenia is reproducible and dose-related. One drug-related death at the maximum tolerated dose was reported in a patient previously treated with radiotherapy.

Mild and transient thrombocytopenia may be observed simultaneously with leukopenia. Anemia necessitating red blood cell transfusion is noted after multiple courses of therapy.

Gastrointestinal intolerance

Approximately 60% of patients experience nausea during drug administration and half of them have minimal vomiting easily controlled by antiemetics. Diarrhea is relatively rare.

Alopecia

Alopecia is commonly encountered, becoming moderate to severe with multiple courses of therapy.

Miscellaneous

Various additional toxicities may occur with insignificant frequency. They include flu-like syndrome, stomatitis and dermatitis. Cutaneous erythema and scaling have been described in previously irradiated areas.

There was no evidence of hepatic, pulmonary, renal or cardiac toxicity in any patient treated with ICRF-159. No significant decrease in serum calcium level has been reported.

CLINICAL STUDIES

Initial therapeutic results with ICRF-159 were reported in 1969 [24]. Surprisingly, since then, only a limited number of disease-oriented studies have been undertaken and thus, little data has accumulated regarding the spectrum of activity of this compound.

Lymphoma

The efficacy of ICRF-159 in lymphoma was detected in early clinical trials [24]. In a phase II study carried out by the Western Cancer Study Group [25], the drug was given in a split dose on

a weekly schedule to 27 patients with far-advanced previously treated non-Hodgkin's lymphomas. Complete responses were achieved in 2 patients for 11+ and 15 months, and partial responses in 6 patients with a remission duration ranging from 0.5+ to 12+ months. In the same study, no response to ICRF-159 was seen in 9 additional patients with Hodgkin's disease; an inadequate dose-schedule of the drug was used and thus its value against this malignancy cannot yet be ascertained.

Colorectal carcinoma

ICRF-159 has been evaluated in advanced colorectal carcinoma with the same daily times 3 schedule by Marciniak *et al.* [20] and Bellet *et al.* [21]. In the first study, no response was seen in 12 patients previously treated with chemotherapy (5-fluorouracil alone or in various combinations). However, in 13 patients not previously exposed to cytotoxic agents, responses were seen in 3 cases. In the second study, all patients had received 5-fluorouracil and some received MeCCNU as single agents. Of the 35 patients evaluable for response to ICRF-159, partial remission was achieved in 5. The median duration of response was 4 months, ranging from 1 to 5 months. None of the responders had previously experienced antitumor effect following systemic chemotherapy with either 5-fluorouracil or MeCCNU.

Lung cancer

Promising results were obtained in early clinical trials with ICRF-159 in squamous cell carcinoma of the lung [26]. These results were not confirmed by a subsequent trial undertaken at the Mayo Clinic [27]. In the latter extensive Phase II study, most of the patients did not receive prior chemotherapy. After stratification for performance status, prior chemotherapy and cell type, patients were randomized to ICRF-159 alone or a combination of vincristine, bleomycin and adriamycin with cross-over after failure to either regimen. Responses to ICRF-159 in squamous cell, large cell and adenocarcinoma of lung were obtained in 1/17, 1/10 and 2/25 cases respectively.

Melanoma

ICRF-159 was evaluated in 20 patients with metastatic melanoma resistant to prior sequential single agent systemic therapy with DTIC and a nitrosourea [19]. No objective response was achieved. In that study, immunosuppressive effects as determined by a lymphocyte microcytotoxicity assay were minimal.

Sarcoma

ICRF-159 has been used for the treatment of 18 patients with Kaposi's sarcoma resistant to prior therapy [28]. One patient exhibited complete tumor regression for 5+ months and 10 had a partial response.

In a pilot study, 22 patients with soft tissue sarcomas and osteosarcomas were treated with a combination of radiation therapy plus ICRF-159 [29]. These tumors are generally radioresistant, but with the combination, 6 patients had complete remissions and 12 had partial remissions. Unexpectedly severe skin reactions in the irradiated fields were reported, reflecting a potentiation of the usual cutaneous toxicity encountered with irradiation alone.

Miscellaneous

Limited studies with ICRF-159 were devoted to trials involving acute leukemia. The preliminary available data warrants further investigation in this disease [24, 30].

Phase I studies have been conducted using ICRF-159 in combination with adriamycin [15] or *cis*-diamminedichloroplatinum [31]. Additional data should clarify whether the usefulness of these combinations, originally detected in animal experiments, might be reflected in clinical practice.

ONGOING CLINICAL TRIALS

Few studies of ICRF-159 as a single agent are presently ongoing. At the Southwest Oncology Group, the drug is being tested in pediatric solid tumors and in acute lymphocytic leukemia. Phase II trials in breast cancer are currently being carried out at the Mayo Clinic; phase II evaluation of ICRF-159 in breast cancer and in squamous cell carcinoma of head and neck are in progress at The Fox Chase Cancer Center. The single agent activity of ICRF-159 is presently also being investigated in pancreatic cancer, lung cancer and sarcoma by several cooperative groups in the United States.

DISCUSSION

ICRF-159 is a cytotoxic antitumor agent with a novel structure; its actual therapeutic potential remains generally uncertain at the present time. This agent has been clinically evaluated only in its oral form with intestinal absorption following a peculiar and incompletely understood pattern [18], underscoring the need for precise pharmacologic data prior to extensive clinical studies of

the efficacy of an oral agent. On the other hand, the oral form of ICRF-159 is subjectively well tolerated and intestinal absorption is apparently reproducible in the same patient. Thus far, the lack of solubility of ICRF-159 as a racemic mixture has precluded its parenteral administration. Recently, the separate use of enantiomers of the compound was shown to overcome this difficulty [32], and an intravenous formulation suitable for clinical testing will be soon available.

It is puzzling that relatively few studies involving ICRF-159 are ongoing. Perhaps its early introduction into many combination studies and the delay in establishing an optimal dose-schedule have hampered full evaluation of the drug. Too many investigational anticancer drugs have been discarded in the past without sufficient rationale [33, 34]. In the particular case of ICRF-159, a number of important signal tumors have not been treated. The necessity of testing drugs in a broad spectrum of tumor types has been repeatedly emphasized [35], and hints of clinical activity detected with ICRF-159 have been sufficiently meaningful to deserve further investigation.

Activity of ICRF-159 has been manifest in colorectal carcinoma [20, 21], probably with no cross-resistance to 5-fluorouracil or MeCCNU [21] (2 other agents of moderate activity in this disease). Its combination with 5-fluorouracil would be worth investigating in view of the reported synergism of this combination in the L1210 system [15, 16]. The prevention of metastases in several experimental tumor systems [9, 10] makes ICRF-159 attractive for adjuvant trials. Although 5-fluorouracil was also found to inhibit metastases in the Lewis lung carcinoma, its value as an adjuvant treatment in colorectal malignancies remains questionable. Adjuvant studies in this disease might be considered with ICRF-159 plus 5-fluorouracil in combination after careful phase I and phase II evaluation.

Other combinations warrant further investigation. Experimental potentiation by ICRF-159 of the antitumor activity of the anthracyclines [14–16], as well as the possible inhibition of drug induced cardiotoxicity [17], makes a combination attractive for clinical study. Initial results obtained with ICRF-159 plus radiotherapy in human sarcoma [29] have pointed to the possible radiosensitizing ability of ICRF-159. These findings need confirmation insofar as the many attempts to improve the effectiveness of radiation therapy by concomitant chemotherapy have to date been disappointing [36].

In conclusion, ICRF-159 is an antitumor agent endowed with many attractive properties. Additional studies are needed to accurately assess the role of this new drug in the treatment of cancer.

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5-S-Cysteinyldopa in Diagnosis and Treatment of Human Malignant Melanomas and Ultrastructural Observations*

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Abstract—*5-S-cysteinyldopa (5-S-CD) was determined in the tumors and urine of* 31 *primary and/or metastatic melanoma patients and* 8 *control subjects. 5-S-CD was present in all but one melanoma. Urinary excretion of 5-S-CD was in the normal range in patients with primary melanoma but often increased in disseminated melanoma. Determination of 5-S-CD in the tumors seems to be of value for the diagnosis of unpigmented tumors primary or metastasis and in the follow-up of treatment of patients with disseminated melanoma.*

There was no evident correlation between secretion of 5-S-CD and the ultrastructure of the tumors.

INTRODUCTION

DOPA and dopamine have been described as frequently occurring catechols in tumors and urine of Syrian hamsters with melanotic tumors [1] and in the urine of patients with malignant melanoma [2–3].

Since then it has been shown that 5-S-cysteinyldopa (5-S-CD), a catechol which plays a role in phaeomelanin production, can be detected in the urine of melanoma patients with tumors and during the evolution of the disease [4–8]. A recent study showed a wide variation in the amount of 5-S-CD in cultured cells and culture medium obtained from established cell lines of human malignant melanocytes [9–12]. Our aim was to determine 5-S-CD levels in primary and/or metastatic tumors and urine of patients with malignant melanoma. We compared these 5-S-CD levels with the ultrastructure of tumors, particularly in respect to the different structural characteristics of pathologic melanosomes [13]. We also tried to ascertain whether there was a relationship between therapeutic treatment and its usefulness for the follow-up of the patients.

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MATERIAL AND METHODS

Thirty-one melanoma patients were investigated. Sex, age, hair colour, presence of freckles and pathologic data following Clark's classification [13] (levels of invasion) are given in Tables 1, 2 and 4.

Biochemical determinations

The presence of 5-S-CD in both the tumors and urine of 29 patients was tested by the method previously described [14].

5-S-CD in the tumors

The excised tumors tested consisted of: 13 primary melanomas, 5 primary tumors and regional lymph nodes, 11 solitary and/or multiple recurrent metastases (cutaneous metastasis and/or lymph nodes). For two patients (cases 28 and 30), 5-S-CD was determined in two different lymph nodes: one pigmented, and one unpigmented (Tables 1 and 2).

Two normal skins, one benign naevus, one

Table 1. *Clinical and morphologic data and* 5-*S-CD levels in patients with primary melanoma*

Patients	Sex	Age	Hair colour	Freckles	Level of invasion*	5-S-Cysteinyldopa		Ultrastructural aspect	
						Tumor μg/g	Urine μg/24 hr	Structure of premelanosomes	Mature melanosomes
A. Lentigo maligna melanoma									
1. MAR.	♀	49	brown	+	III	7	107	granular	+
2 ALA.	♀	75	grey	+	V	36	58 (M)	fibrillar	+ +
B. Superficial spreading melanoma									
3 GUI.	♀	73	grey	+	III	6	74	fibrillar	
4 DUB.	♀	34	black	+	IV	22	167	fibrillar	+
5 BOE.	♀	82	grey	+	V	0	527 (M)	fibrillar	+ +
6 COU.	♀	49	brown	+	V	29	1526 (M)	fibrillar	±
7 ROP.	♀	68	grey	−	V	2	—	fibrillar	±
C. Nodular melanoma									
8 COU.	♀	58	grey	−	III (A)	2	29 (M)	network	+
								structure	±
9 CLA.	♀	50	blond	+	III	139	—	granular	
10 COL.	♀	67	grey	−	III	83	31	granular	+
11 THE.	♂	62	grey	+	IV	69	168 (M)	granular	±
12 COV.	♀	57	brown	−	IV	110	86	granular	−
13 QUI.	♂	25	red	+	V	84	163	granular	−
14 ANG.	♂	40	blond	+	V	155	236	granular	±
15 ROS.	♀	29	brown	−	V	519	228 (M)	fibrillar	+ +
16 TAR.	♀	28	blond	+	V	225	22 (M)	fibrillar	±
D. Non cutaneous melanoma (mucosae)									
17 TOU.	♂	64	grey	−	nasal mucosa	21	63	fibrillar	+
18 COR.	♂	78	white	−	rectum mucosa	11	109 (M)	fibrillar	+

(M) = With metastasis. (A) = Unpigmented melanoma. *According to Clark *et al.*

Table 2. Clinical and morphologic data and 5-S-CD levels in patients with metastatic melanoma

Patients	Sex	Age	Hair colour	Freckles	Pigmentation of tumors*	5-S-Cysteinyldopa		Ultrastructural aspect	
						Tumor μg/g	Urine μg/24 hr	Structure of premelanosomes	Mature melanosomes
A. Cutaneous metastasis									
19 ALA.	♀	75	grey	+	+	29	85 (P)	fibrillar	+ +
20 PRO.	♀	57	blond	+	+	4	149		NT
21 SUK.	♂	61	white	+	+	28	240	fibrillar	+ +
22 JAM.	♀	41	brown	+	+	72	70	granular	+ +
23 MAZ.	♂	66	grey	−	+	1	219	fibrillar	+ +
24 BOE.	♀	82	grey	−	+	0	527 (P)	fibrillar	+ +
25 ROD.	♂	36	blond	+	−	1	274	network structure	−
26 CAR.	♂	43	blond	+	+	168	24885	fibrillar	+ +
27 ESQ.	♀	43	brown	−	+	51	2218	fibrillar	+
B. Lymph nodes metastasis									
28 VIL.	♂	46	black	+	+	75	284	fibrillar	±
					−				
29 SUK.	♂	61	white	+	+	150	240	fibrillar	+
30 BON.	♂	74	grey	+	+	110	188	granular	+
					−	< 1			
31 ARI.	♂	39	brown	+	+	2	286	fibrillar granular	±
32 ESQ.	♀	43	brown	−	+	425	2218	fibrillar	+
33 THE.	♂	62	white	+	+	14	169 (P)	NT	NT
32 TAR.	♀	28	blond	+	+	7	22 (P)	fibrillar	+
35 BAU	♂	64	black	−	+	144	450	fibrillar	+ +
36 COU.	♀	58	grey	−	−	< 1	29 (P)	granular	−
37 BOE.	♀	82	grey	−	+	< 1	51 (P)	fibrillar	+

(P) = With primary melanoma; *(+ = pigmented melanoma, − = unpigmented melanoma); NT = non-tested.

Table 3. 5-S-CD and dopa + dopamine levels in tumors and urine of controls

Patients	Sex	Age	Hair colour	Freckles	Diagnosis	5-S-cysteinyldopa Tumor μg/g	5-S-cysteinyldopa Urine μg/24 hr
A. In tumor controls and normal skin							
1 YVA.	♀	27	brown	−	normal skin	0	197
2 DUR.	♂	65	grey	−	normal skin	0	−
3 BAS.	♀	14	dark	−	naevus	0	—
4 SAR.	♀	40	brown	+	pigmented basal cell carcinoma	0	59
5 SAN.	♂	70	white	+	squamous cell carcinoma	0	—

B. In urine controls						
Patients	Sex	Age	Hair colour	Freckles	5-S-Cysteinyldopa μg/24 hr	Dopa + dopamine μg/24 hr
1 GIR.	♂	66	grey	+	4	22
2 MOR.	♂	39	brown	−	40	558
3 SAR.	♀	40	brown	+	59	—
4 CUL.	♀	70	grey	−	67	114
5 CAS.	♀	50	brown	+	136	150
6 CHA.	♂	32	brown	+	157	338
7 YVA.	♀	27	brown	−	197	—
8 BON.	♂	74	grey	+	94	80

pigmented basal cell carcinoma, and one squamous cell carcinoma, were used as controls (Table 3).

5-S-CD in the urine

The urine of 31 patients were tested for 5-S-CD. For the 22 patients with primary or/and metastatic the level of 5-S-CD was determined immediately before surgical treatment. For 5 patients the urinary excretion of 5-S-CD was followed up monthly. For 3 patients (cases 2, 5 and 6) with loco regional disease we followed up the evolution levels in the urine during surgical and immunochemotherapic treatment (BCG + DTIC) (Fig. 1). For two patients with widespread lesions (cases 26 and 57) the monthly determinations were carried out up till death (Fig. 2). 5-S-CD determinations were also carried out on the urine of 8 control subjects (Table 3).

In addition dopa + dopamine levels were studied in 5 tumors and in the urine of 11 primary or metastatic melanoma patients (Table 4) according to Anton and Sayre [15].

Electron microscopy

All the tumor fragments were processed for electron microscopy by the classical method glutaraldehyde-osmium fixation, dehydration in alcohols and embedding in epon [16]. The sections obtained with an ultramicrotome Reichert (MT2) were counterstained with uranyl acetate and lead citrate [17] and observed with a Jeol electron microscope JEM 100C.

RESULTS

Biochemical results

In melanoma tumors. As seen from the Tables 1 and 2, a great variability in 5-S-CD levels was found in the tumors (primary and metastatic). It is of interest to note that the average level was higher in nodular melanoma than in superficial spreading melanoma.

It is important to note the presence of 5-S-CD in unpigmented tumors (cases 8, 25, 28, 30 and 36).

There was no correlation between 5-S-CD levels in the tumors and hair colour and freckles for the same patient.

In the control tumors. In normal skin and other cutaneous tumors, 5-S-CD was not detected (Table 3).

Varying amounts of dopa + dopamine were found in the tumors of 5 patients. Dopa was present in the absence of 5-S-CD in only one case (case 38) which was a highly pigmented tumor.

In urine. Healthy controls: The amount of 5-S-CD in the urine of eight healthy control subjects varied between 4 and 197 μg/24 hr. Dopa + dopamine varied between 22 and 558 μg/24 hr (Table 3).

Patients with primary melanoma: In patients with primary melanoma who had no signs of metastasis, the level of 5-S-CD ranged from 31 to 236 μg/24 hr were within the normal range, even when the amount of 5-S-CD in the tumor was high (Table 1).

Table 4. 5-S-CD and dopa + dopamine levels in tumors and urine of patients with melanoma

A. In tumors

Patients	Sex	Age	Hair colour	Freckles	Tumors	5-S-Cysteinyldopa µg/g	Dopa + dopamine µg/g
38 BOE.	♀	82	grey	−	primary	0	0.6
39 COU.	♀	49	brown	+	primary	29	1.6
40 ROS.	♀	29	brown	−	primary	519	6.5
41 ARI.	♂	39	blond	+	metastasis	2	0.2
42 BON.	♂	74	grey	+	metastasis	110	2

B. In urine

Patients	Sex	Age	Hair colour	Freckles	Tumors	5-S-Cysteinyldopa µg/24 hr	Dopa + dopamine µg/24 hr
43 TOU.	♂	64	grey	−	primary	63	217
44 TIA.	♂	48	brown	+	solitary Reg. l.n.	108	201
45 BAU.	♂	64	black	−	Reg. l.n.	169	147
46 BON.	♂	80	grey	−	primary	188	376
47 ROS.	♀	29	brown	+	primary	228	180
48 SUK.	♂	61	blond	+	Reg. l.n.	240	151
49 ROD.	♂	36	blond	−	multiple subcutaneous Reg. l.n.	274	620*
50 BOE.	♀	82	grey	+	solitary Reg. l.n. solitary cutaneous	527	318
51 COU.	♀	49	brown	+	primary	1526	274
52 CAS.	♂	52	brown	+	gastrointestinal	12000	564
53 CAR.	♂	43	blond	+	multiple subcutaneous and cutaneous	44586	568†

Reg. l.n. = Regional lymph nodes; * = faintly pigmented; † = heavily pigmented.

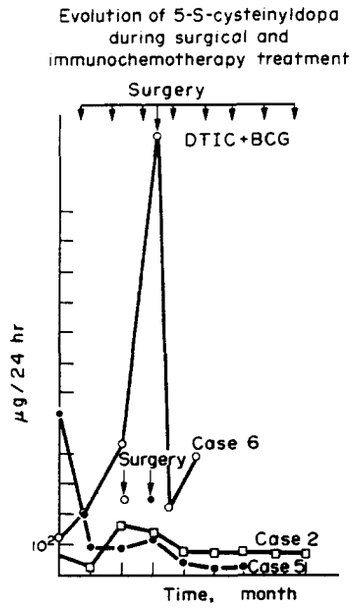

Fig. 1. Regional recurrence. Immunochemotherapy + surgical treatments.

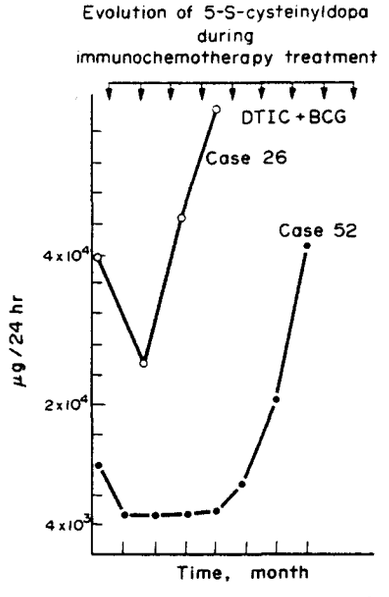

Fig. 2. General recurrence. Immunochemotherapy.

Patients with metastasis: in patients with primary melanoma and loco-regional metastasis (Table 1 (M)), 2 of the 8 had pathologically increased values of 5-S-CD (cases 5 and 6) while 6 patients had values from 29 to 228 µg/24 hr which were within the normal range.

In 6 patients with only recurrent disease (4 pigmented and 2 unpigmented tumors) we considered that the 5-S-CD urine values were definitely pathological (Table 2).

There was no correlation between 5-S-CD levels in the tumors and in the urine particularly when very high levels in the tumors were accompanied by low levels in the urine (cases 15 and 16) and inversely (case 6) even when the total tumor mass is taken into account (Table 1).

After treatment

Surgical treatment results in a decrease of the amount of 5-S-CD (Fig. 1). In case 6 the 5-S-CD level after surgery shows that all the tumor had not been excised as compared with cases 2–5 where there was complete excision.

During immunochemotherapy treatment, we observed (Figs. 1 and 2): First, a persistent increase in the urine 5-S-CD levels which indicated the inefficiency of the therapy (case 6). Secondly, after a decrease for a short time, the rapid increase in 5-S-CD showed a failure of the treatment (cases 2–26). Thirdly, a decrease with stabilization in the amount of 5-S-CD which showed that treatment was effective (cases 2–52). Lastly, when the treatment was not effective and or new metastases occurred, there was a subsequent increase in the urine level (cases 6, 26 and 52).

Dopa + dopamine levels in the urine were variable.

Electron microscopy

The premelanosome structures and presence of mature melanosomes appear in Tables 1 and 2. The specific cell structures and the morphology of the melanosomes are shown in Figs. 3–10.

In the tumors (primary and metastasis) we observed 4 typical structures of pathologic melanosomes [18, 19]: fibrillar (Fig. 3); granular (Fig. 4); lamellar organelles (Fig. 5); and "network structures" (Fig. 6).

From Table 1 it can be seen: in the primary tumors, we observed for lentigo maligna melanomas, for superficial spreading melanomas and non-cutaneous melanomas that the premelanosomes were often fibrillar. The premelanosomes of nodular melanomas were often granular. For the metastasis, different types of premelanosomes were observed but the fibrillar structure was more frequent. The different morphology of the mature melanosomes (oval-shaped (Figs. 7 and 8) and round (Figs. 9 and 10) were compared with 5-S-CD tumor levels, and no correlation appeared. There was also no correlation between hair colour and the melanosome morphology in the tumors.

DISCUSSION

5-S-CD determination in tumors is related to the presence of malignant melanocytes but is not

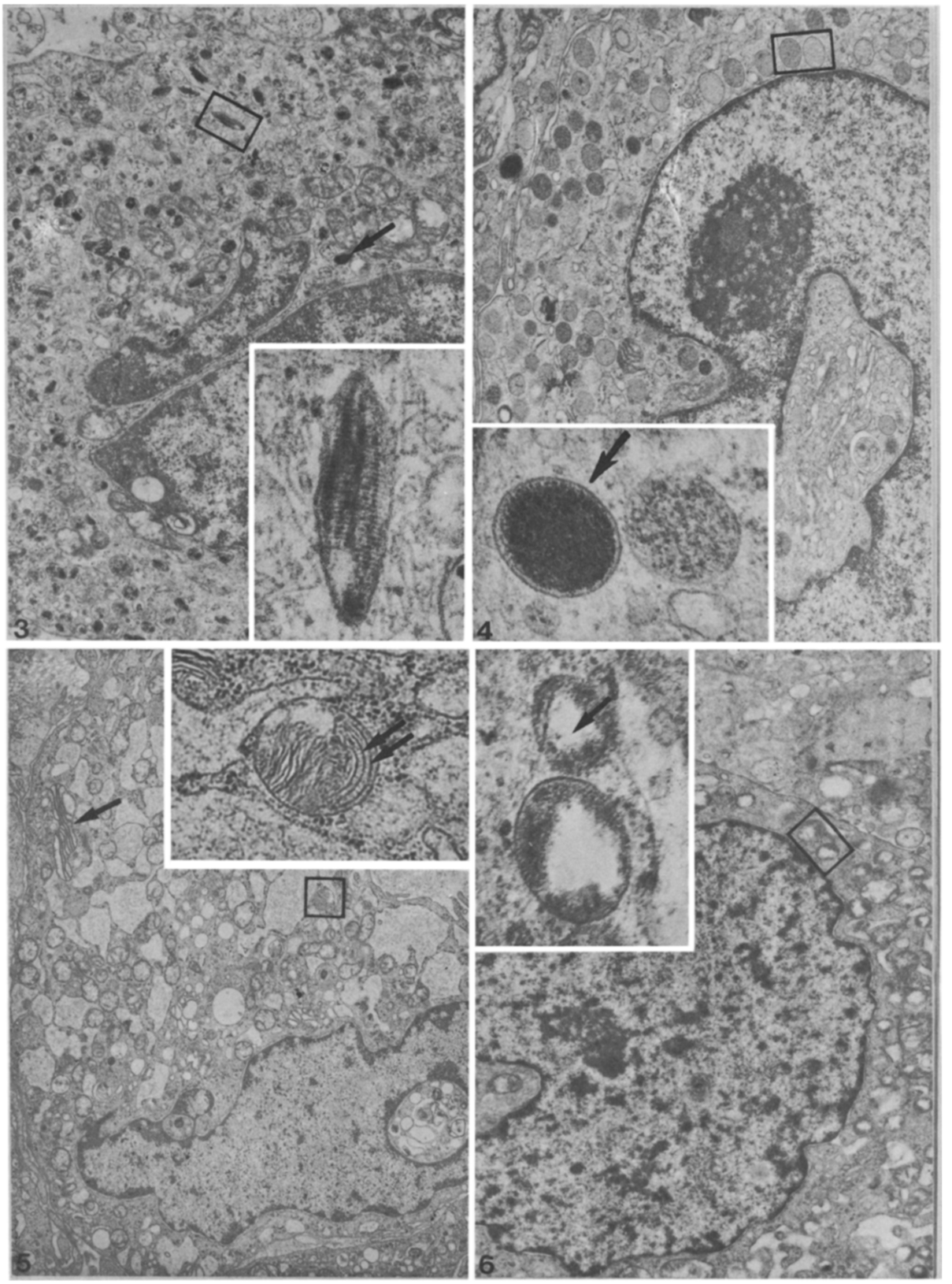

Fig. 3. Case No. 15. Mucosa melanoma: Very differentiated melanocytes showing complete mature (→) and typical fibrillar melanosomes, the structure of which is detailed in the inset. (Mag. × 15,000; inset mag. × 100,000).

Fig. 4. Case No. 21. Cutaneous metastasis. Melanocytes with typical granular melanosomes bound by the trilaminar membrane. (Mag. × 10,000; inset mag. × 50,000).

Fig. 5. Case No. 25: lymph node metastasis: poorly differentiated cell showing a pluri-infolded nucleus. Numerous mitochondria are present, some with a very characteristic appearance (→). Lamellar organelles are present, the detail of which, shown in the inset, revealed a striated structure (⇉). (Mag. × 8000, inset mag. × 50,000).

Fig. 6. Case No. 14. Primary tumor: nodular melanoma completely dedifferentiated cell showing network structures. They are single membrane-bound organelles with the same size as the granular melanosomes. They present a dense zone at the periphery and a clear central zone more or less criss-crossed by a fine network (→). (Mag. × 8750; inset mag. × 50,000).

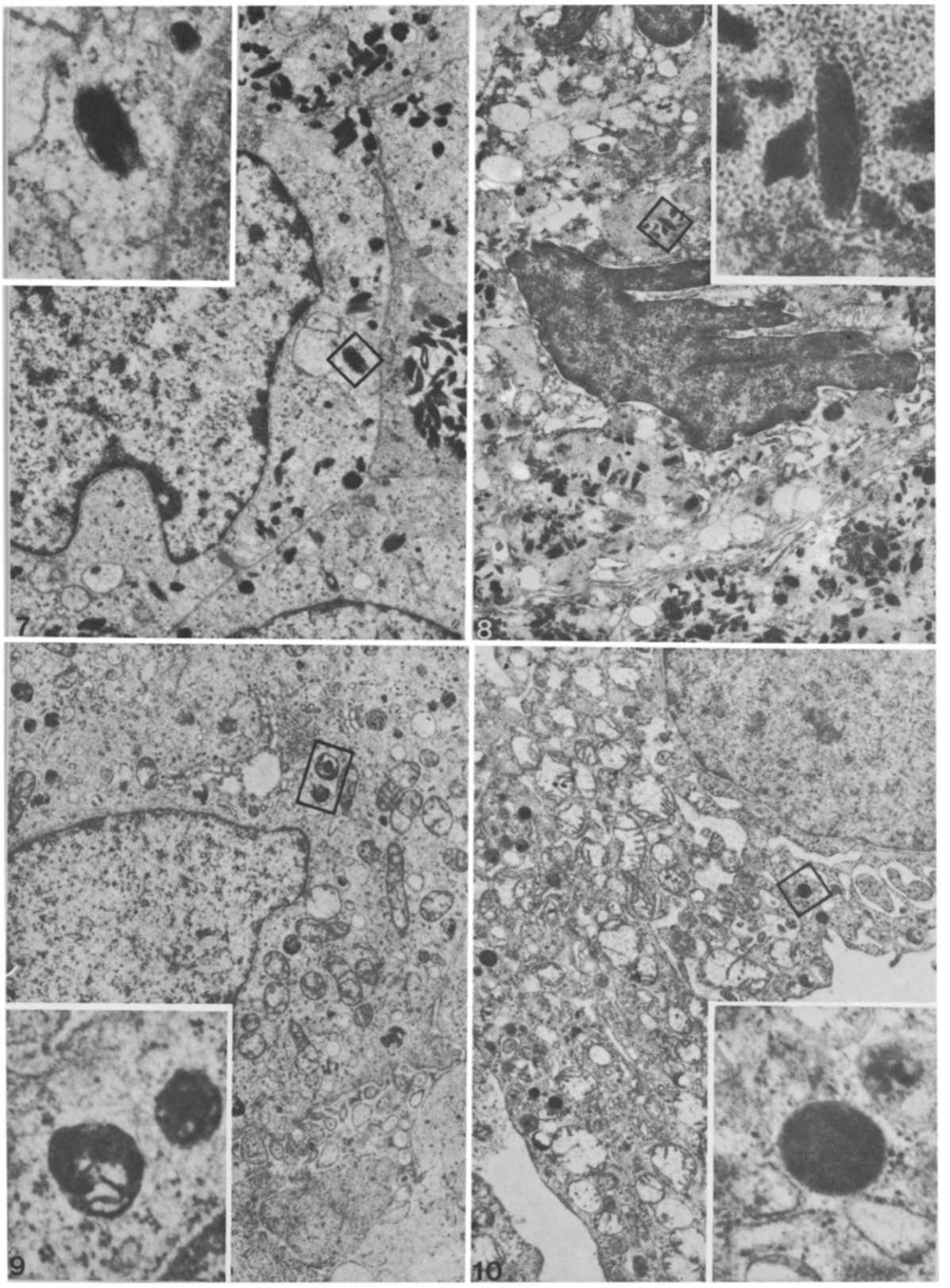

Fig. 7. Case No. 32. Lymph node metastasis. Malignant melanocyte with oval-shaped fully melanized melanosomes. The tumor was from a fair haired patient and produced no 5-S-CD. (Mag. × 1000; inset mag. × 68,000).

Fig. 8. Case No. 3 Primary tumor: superficial spreading melanoma. Fully melanized oval-shaped melanosomes. The tumor was from a fair haired patient and produced no 5-S-CD. (Mag. × 1000; inset mag. × 68,000).

Fig. 9. Case No. 25. Lymph node metastasis. Malignant with round, sometimes incompletely melanized melanosomes. The tumor was from a black haired patient and produced a high level of 5-S-CD. (Mag. × 12,000; inset mag. × 50,000).

Fig. 10. Case No. 24. Cutaneous metastasis. Typical round melanosomes in tumor cells from a fair haired patient. This tumor had a high level of 5-S-CD. (Mag. × 10,000; inset mag. × 56,000).

specific since this phaeomelanin metabolite is also found in some pigmented naevi but in much lower amounts [20].

On the contrary, we did not find 5-S-CD present in normal skin or in other types of cutaneous tumors, even when they were pigmented. Therefore, a substantial level of 5-S-CD in a tumor favors the diagnosis of malignant melanoma.

Moreover, positive 5-S-CD determinations allow the diagnosis of melanoma to be made for unpigmented tumors which are most difficult to diagnose [cases 8 (Table 1), 25, 31 and 36 (Table 2)].

No correlation between 5-S-CD levels and the tumor pigmentation was found. Some very heavily pigmented tumors had very small amounts of 5-S-CD. This is due to the fact that phaeomelanin gives a fainter pigmentation than eumelanin [21].

It should be noted that 5-S-CD is found in greater amounts in nodular melanoma, which has a poor prognosis, than in superficial spreading melanoma or lentigo maligna melanoma which have a relatively better prognosis. We suggest a possible importance of an admixture of connective tissue in superficial spreading melanomas and in lentigo maligna melanomas to be responsible for the lower 5-S-CD values.

Nevertheless, we cannot affirm that the amount of 5-S-CD in the tumor has any prognostical value.

The wide variability in the amounts found in the tumors suggests that the proportion of pathologic melanocytes able to synthesize 5-S-CD varies from one tumor to another one.

The absence of a correlation between 5-S-CD levels in the tumors and in the urine suggests that 5-S-CD production and excretion are not always related.

There was no evident relationship between the structure of the premelanosomes and the level of 5-S-CD. We noted that the morphology of the melanosomes in the tumors did not correspond to those that Parakkal [22] described in normal skin of blond and black-haired people. These results suggest either that melanosome shape does not always determine hair color, or that these patients do not present the same melanosome shapes in their normal skin or tumors.

So, the determination of 5-S-CD in the urine of the patients with primary tumors and with small metastases is of little interest.

A great variability in the amounts of 5-S-CD excreted in urine by patients was expected as a wide variation had been observed in healthy subjects [23] particularly after exposure to the sun [24].

In the tumor of one patient where 5-S-CD was absent, dopa + dopamine were present whereas the 5-S-CD level in the urine was pathologic which suggests an abnormality in the secretion of this metabolite.

However, 5-S-CD determinations are of great value in the case of widespread disease. In these cases, the 5-S-CD determinations allow the evaluation of the therapeutic treatment for immunochemotherapy. Thus, not only can post-surgical strategy be better decided, but the follow-up therapy and time of treatment can be adapted as required. Evidently, a sudden increase in the 5-S-CD level is an indication for instigating another treatment.

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Urinary Excretory Patterns of tRNA Degradation Products: A Marker of Hodgkin's Cell Metabolism?

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Abstract—*Aberrant tRNA methylase activity has been described in a significant variety of animal tumours of both viral and chemically-induced aetiology. The possibility has been entertained that this may result in, or be the result of, altered metabolism of tRNA, with increased turnover of this molecule being mirrored by the appearance of degradation products in the plasma and urine. It is also possible that the tRNA may become hypermethylated. Hodgkin's disease, while possessing a variety of histologic types, has distinctive cells which may be present in either small or large numbers. The clinical prognosis would appear to relate to this histologic variation. This paper presents observations in 96 patients on the urinary excretion pattern of tRNA degradation products, pseudouridine and methylated guanines and demonstrates the strongly significant relationship of increased levels of these compounds to the number of Hodgkin's cells (atypical histiocytes). In addition, it relates these observations to the significance of the constitutional symptoms of the disease.*

INTRODUCTION

A NOTABLE feature of tumour metabolism is the presence of aberrant tRNA methylase activity. Increased activity of these enzymes has been reported in several tumours and there is evidence of hypermethylation of tRNAs extracted from solid tumours [1]. We have found increased amounts of methyl guanosine in tRNA from some tumours [2], but it has been difficult to correlate methylation of tRNA with hyperactivity of the methylating enzymes themselves.

There is evidence of increased excretion of tRNA components, methylated purines, and pseudouridine, in patients with various neoplastic diseases [3–8]. We have made similar observations on elevated pseudouridine levels in the urine of untreated newly diagnosed patients with Hodgkin's disease [9]. A relationship between this and the presence of constitutional symptoms was also observed and it was suggested that this may relate to increased metabolic activity of the Hodgkin's cell (atypical histiocyte). Although some elevation of methyl purines was also observed, this was not as consistent as the results on excretion of pseudouridine. Since then, we have studied a further 96 patients with newly diagnosed Hodgkin's disease and have used more sensitive methods for quantitation. This report relates the levels of methylated guanines to other features of the disease.

MATERIAL AND METHODS

Twenty-four-hour urine specimens were obtained from 96 patients with newly diagnosed Hodgkin's disease. The patients were placed on a low purine diet for 4 days and urine was collected on the 4th day. An aliquot (2.5% of total volume) was prepared and analysed according to a previously described method [9]. However, on this occasion more sophisticated instrumentation using a Gilford scanning spectrophotometer facilitated identification and quantitation of the various major and minor bases. The 1-methyl and 7-methyl guanine were grouped together and 8-OH-7-methyl guanine was estimated separately. Histiocytes were scored by assessing the percentage of such cells in biopsy specimens. tRNA methylase activity of tumour tissue was assayed as previously de-

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scribed [2]. The methylase assays were carried out in only 12 of the 96 patients as tumour material was available in only this number of the patients included in this study.

RESULTS

Among the 96 patients studied, 50 demonstrated a high pseudouridine excretion (Table 1). Of these patients, 22 also had elevated levels of methylguanine (Table 2). It was of interest to note that in the group of 46 patients with normal pseudouridine levels, 42 had normal or undetectable amounts of methyl guanines, whereas only 4 had above normal levels of excretion. A similar observation was also made in the case of 8-OH-7-methyl guanine (Table 3), both observations being highly significant.

The relationship between elevated levels of pseudouridine and the presence of constitutional symptoms was statistically highly significant (Table 1). Constitutional symptoms were present in 76% of patients with elevated pseudouridine levels but only in 39% of those with normal or reduced levels. The correlation between elevated pseudouridine levels and the number of atypical histiocytes (Hodgkin's cells) present in biopsy specimens was also significant (Table 4). Sixty-eight percent of patients with elevated levels had scores above 20% of

Table 1. *Relationship between* 24 *hr urinary excretion of pseudouridine and presence of constitutional symptoms*

Pseudouridine levels	Constitutional symptoms Present		Absent		Total No. of patients
>Normal*	38	(76%)	12	(24%)	50
Normal or <Normal	18	(39%)	28	(61%)	46
Total	56	(58%)	40	(42%)	96

$P = < 0.001$

Constitutional symptoms = fever, night sweats, weight loss of 10% body weight.

*Normal urinary pseudouridine levels = 48–88 mg/24 hr.

Table 2. *The relationship between pseudouridine and* 1 *and* 7*-methyl guanine levels in* 24 hr *urine excretion*

Pseudouridine levels	Excretion of 1 and 7-methyl guanines Elevated		Normal† or not detected		Total No. of patients
>Normal	22	(44%)	28	(56%)	50
Normal or <Normal	4	(7)	42	(93%)	46
Total	26	(27%)	70	(73%)	96

$P = < 0.001$

† Normal urinary 1 and 7-methyl guanine levels = 3–7 mg/24 hr.

Table 3. *The relationship between pseudouridine and* 8*-OH-*7*-methyl guanine levels in* 24 hr *urine excretion*

Pseudouridine levels	Excretion of 8-OH-7-methyl guanine Elevated		Normal‡ or not detected		Total No. of patients
>Normal	22	(44%)	28	(56%)	50
Normal or <Normal	2	(4%)	44	(96%)	46
Total	24	(25%)	72	(75%)	96

$P = < 0.001$

‡ Normal urinary 8-OH-7 methyl guanine levels = 0–3 mg/24 hr.

Table 4. Correlation between presence of "atypical" histiocytes and 24 hr urinary excretion of pseudouridine

Pseudourine levels	No. of patients with atypical histiocytes Score >20%	Score <20%	Total No. of patients
>Normal	34 (68%)	16 (32%)	50
Normal or <Normal	19 (41%)	27 (59%)	46
Total	53 (55%)	43 (45%)	96

$P = < 0.025$

Atypical histiocytes = a term applied to the mononuclear Hodgkin's cells—and does *not* include Reed–Sternberg cells.

Table 5. Correlation between hyperactive tRNA methylases and elevated pseudouridine levels

No. of patients with elevated urinary pseudouridine levels	tRNA methylase activity Increased	*Normal
12	11	1

*Normal range values for ^{14}C H_3 incorporation = 410–1037 counts/min/100 μg tRNA.

Hodgkin's cells, whereas 41% had a negative correlation.

DISCUSSION

This report confirms our earlier observations on increased excretion of pseudouridine and methylated bases in patients with Hodgkin's disease [9]. However, we are now better able to measure the levels of excretion of previously undetectable amounts of bases, due to the increased sensitivity of the present methods of quantitation. A highly significant number of patients have been shown to excrete abnormal amounts of 1 and 7-methyl guanines and 8-OH-7-methyl guanine, the latter being a degradation product of 7-methyl guanine.

Observations on increased excretion of pseudouridine and methylated purines have also been made in patients with leukaemia [3, 4], polycythaemia vera, gout and psoriasis [5, 6] and mental retardation [10]. More recently Waalkes showed increased excretion of pseudouridine, 1-methyl inosine and N^2N^2-dimethylguanine in patients with various neoplastic diseases [8, 11–13], and Ho and Lin [14] observed increased excretion in hepatomas of 1 and 7-methyl guanine, dimethyl guanine, 1-methylhypoxanthine and adenine.

Methylated nucleic acid bases and pseudouridine are found predominantly in tRNA [15], hence their presence in serum and urine is presumed to be due to degradation of tRNA. Methylation occurs after formation of the macromolecule [16–17], but after degradation, these compounds are not reincorporated into the tRNA structure [17]. Similarly, free pseudouridine is not synthesized into the tRNA molecule [18]. Elevated levels of excretion of methylated nucleosides could therefore suggest increased tRNA methylase activity and possibly tRNA turnover, if pseudouridine were also elevated.

The origin of methylated bases from degradation of tumour tRNA in particular has not been entirely proven. We have observed increased activity of tRNA methylases in Hodgkin's tumours of 11 out of 12 patients (Table 5) who also had increased urinary excretion of pseudouridine. This suggests that there is at least increased methylation occurring in the Hodgkin's tissue.

It would appear that altered metabolism is taking place in the Hodgkin's cell rather than in the tumours in general as such alteration is most common in those who have the highest percentage of Hodgkin's cells in tumour tissue. Studies are in progress to isolate tRNA from Hodgkin's tissue and to analyse its base composition in an attempt to obtain further evidence of aberrant methylation in the tumour cell.

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Transplantability of Neuraminidase-Treated Ascites Tumour Cells

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Abstract—*Landschütz ascites tumour cells exposed* in vitro *to neuraminidase (VCN) were tested for viability, sialic acid depletion and subsequent tumour development in three mouse strains. Treatment with* 25 *U VCN*/10^7 cells/ml *released* 50% *of the total cellular sialic acid content and no further release was found using a* 20 *fold higher enzyme concentration at* pH 5.5. *The treated cells were normally viable as assessed by vital dye exclusion. VCN reduced tumour transplantability at certain inoculum levels but above a particular tumour dose for each mouse strain no such effect was observed.*

Sera from the three mouse strains used showed no cytotoxic activity in vitro *against untreated or VCN-treated cells.*

INTRODUCTION

EXPOSURE of tumour cells to VCN (neuraminidase from *Vibrio cholerae*) can have three distinct effects on subsequent growth of the tumour *in vivo* [1]. These are (i) decreased transplantability into normally susceptible host animals, (ii) stimulation of the host to reject later challenge with untreated tumour and (iii) immunospecific rejection of established tumours by injection of VCN-treated cells. Altered immune reactivity, resulting from removal of negatively changed sialic acid from the cell surface, is considered to be responsible for these effects but the exact mechanisms involved are still unknown.

While the immunostimulatory effects of VCN-treated cells and their role in rejection of established tumours appear to be well authenticated, considerable controversy exists as to whether VCN effects transplantability [1–3]. Decreased tumour development *in vivo* following *in vitro* exposure of the cells to VCN has been reported by various authors [4–10] while others [11–14] have failed to find such an effect. A possible source of error in assessment of transplantability changes has been pointed out [1, 12]. Sub-optimal conditions of pH or cell density during enzyme treatment can lead to diminished viability of the VCN-treated cell suspension, so that the relevant hosts receive less than the tumour LD_{100}. Furthermore, the accuracy of dye exclusion methods as tests for reproductive integrity has recently been called in question [15].

Apart from viability considerations, other factors which could contribute to the divergent findings in the literature are:
(a) variations in enzyme treatment conditions: for instance, VCN concentrations ranging from 10 to 500 U/ml and pH levels ranging from 5.5 to 7.4 have been used; (b) the fact that not all authors measured release of sialic acid; (c) the presence or absence in the host sera of factors preferentially cytotoxic towards VCN-treated cells; (d) the size of the tumour inoculum used for transplantation, a factor which is not usually taken into consideration and often comprises a single arbitrarily chosen dose.

The aim of the present study was to examine sialic acid removal from Landschütz ascites cells exposed to low or high concentrations of VCN, the viability of these cells and their subsequent growth in Schofield, BDF/1 and A2G mice in response to a wide range of inoculum levels. It will be shown that exposure to VCN concentrations of either 25 U/10^7 cells/ml at pH 7.4 or 500 U/2×10^7 cells/ml at pH 5.5 (accompanied by multiple washing) released 50% of the total cellular sialic acid, while leaving viability unaltered. When certain medium-sized tumour inocula were administered the incidence of takes in Schofield and BDF/1 mice was found to be reduced by VCN. No such reduction in tumour takes was observed using A2G hosts. In all three strains tumour developed normally from VCN-

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treated cells when higher inoculum levels were administered. We also report the absence from the sera of these mice of cytotoxic activity against untreated or VCN-treated cells.

MATERIAL AND METHODS

Mice

Schofield albino (bred as a closed strain since 1967) and BDF/1 (C57 × DBA) mice were obtained from the Medical Research Council of Ireland Laboratories, Dublin. A2G strain were bred in this Department from a nucleus kindly presented by the M.R.C. Animal Laboratory Centre, Carshalton, Surrey.

Tumour

Landschütz ascites tumour, generously donated by Glaxo Research Ltd., Greenford, Middlesex, was passaged in Schofield mice by weekly intraperitoneal transfer. Seven to nine day tumour cells were washed twice in Tris-buffered saline (TBS) and suspended in this medium for use. The medium contained 25 mM Tris, 160 mM Na and 6 mM K and had a pH of 7.4.

Enzyme

VCN was obtained from Calbiochem and from Behringwerke AG as vials containing 500 U in 1.0 ml and used as specified. In each case a unit (U) is defined as the amount required to release 1 μg of *N*-acetylneuraminic acid from a glycoprotein substrate in 15 min at 37°C.

Incubation of cells

Two different conditions of treatment were used.

Procedure (*A*). VCN (Calbiochem) was diluted in TBS and added to twice-washed cells to give a final concentration of 25 U VCN/10^7 cells/ml at pH 7.4. TBS replaced VCN in controls. Incubation was at 37°C for 60 min in a shaking water bath, after which the suspensions were centrifuged at 500 ***g*** for 3 min. The cells were resuspended in TBS, assessed for viability and diluted in TBS when necessary for *in vivo* transplantability studies. The supernatants were retained for sialic acid estimation.

Procedure (*B*). The conditions of Currie and Bagshawe [6] were followed. The cells were washed 6 times in TBS and suspended in the undiluted enzyme preparation which consisted of 500 U VCN (Behringwerke) dissolved in 1 ml 0.05 M sodium acetate, pH 5.5, containing 9 mg NaCl and 1 mg $CaCl_2$. Final concentrations were 500 U VCN/2×10^7 cells/ml. Control samples were suspended in this medium without VCN and otherwise received identical treatment. After incubation at 37°C for 30 min the cells were washed 6 times in TBS, viability assessed and the suspensions appropriately diluted for injection into A2G mice. Supernatants from the incubations and combined supernatants from the subsequent washes were retained for sialic acid estimation.

Cell counts and viability

Counting was carried out in a Burker cytometer under phase contrast microscopy. Viability was determined by vital dye exclusion, using lissamine green and/or trypan blue in final concentration of 0.2 g/l TBS. Testing was carried out immediately after treatment, before dilution of the suspension for inoculation, using a minimum of 5×10^6 cells/ml. Thus the errors inherent in the use of overdilute suspensions [12] were avoided.

Sialic acid

Total cell sialic acid was determined on 2 $\times 10^7$–1×10^8 thrice washed cells. They were digested with 2 ml of 0.1 N HCl at 80°C for 1 hr, centrifuged to remove debris and deproteinised overnight at 4°C with 5% TCA. The dried supernatants were redissolved in 0.5 ml distilled water and free sialic acid estimated. The free sialic acid in supernatants from treated cells was determined after deproteinizing and drying as above. Two methods were used for free sialic acid. (i) Warren's [16] colorimetric assay which has a range of 2–10 μg; in this case the correction for colour produced by deoxyribose was applied (ii) the fluorimetric assay of Murayama *et al.*, [17] which is more sensitive, with a range of 0.1–10 μg. Results were identical using either method. A range of standards (*N*-acetylneuraminic acid, Sigma) was incorporated in all experiments.

Tumour transplantability

Each mouse received a 1.0 m intraperitoneal injection of the specified number of cells. When high cell numbers were used, tumour was allowed to develop for 7 days at which stage the animals showed obvious abdominal distension. With lower inoculum levels 7-day yields were too small for accurate weight determinations, so the growth periods were prolonged to 14–18 days. At the end of the specified time the mice were killed and the peritoneal tumour suspensions collected in TBS. Microscopic examination showed that contamination with white or red blood cells was never more than 8%. Transplantability was assessed as the number of tumour "takes" in proportion to the number of animals injected,

mice yielding ascites cells from the peritoneal cavity being designated as "takes". Neither in "takes" nor "non-takes" was there any evidence of solid tumour formation in the abdominal organs or at the injection site. Survival experiments were also performed, the mice being left until their abdominal cavities were grossly distended and then killed for humanitarian reasons. Post-mortem examination showed the distension to be due to accumulation of ascites tumour: there was no evidence of solid tumour formation.

Tumour yield

Cells obtained from mice which developed tumour were centrifuged at 500 ***g***, dried overnight at 104°C and dry weights recorded. The mean dry weight of cells from those mice in which the tumour grew was taken as an indication of tumour growth.

Serum cytotoxicity

Fresh serum samples, pooled from 3 mice of each strain, were tested for direct cytotoxicity against tumour cells which had been exposed to TBS or VCN according to procedures (A) or (B). Following the method of Sethi and Brandis [9] 10^7 cells/ml TBS were mixed with an equal volume of test serum, either undiluted or diluted 1:1 in TBS, and incubated at 37°C for 45 min without shaking. Cell viability was then tested using lissamine green and trypan blue.

RESULTS

Viability (dye exclusion)

Numerous tests using both lissamine green and trypan blue showed the viability of cells exposed to procedure (A) to be always greater than 90%. Readings for VCN-treated cells never differed from those of controls by more than 5% and these minor differences showed no consistent trend. Procedure (B), which involved extra manipulations and a low pH, resulted in mean viabilities of 80% for controls and 83% when VCN was used. This difference is not significant.

Sialic acid release

The total sialic acid content of untreated Landschütz tumour cells was 471 ± 14 nmole/10^9 cells (mean ± S.E.M. of 19 determinations). The free sialic acid released from treated cells was almost identical under both procedures, being 50% and 49% of the total cellular content in the cases of (A) and (B) respectively (Table 1). In the case of control cells, however, a marked difference was found in the degree of free sialic acid depletion between the two procedures; procedure (B), presumably due to the numerous washes involved, resulted in a loss of 28% of total cell sialic acid, while the corresponding value for cells subjected to procedure (A) was only 6%.

Transplantability

Cells treated according to procedure (A). Table 2 shows the incidence of tumour takes and the mean weights of tumour/mouse (for those animals in which tumour developed) resulting from administration of various inoculum levels to 3 strains of mice. Prior exposure to VCN reduced transplantability when certain cell doses were used. For example, Schofield mice receiving 10^5, 10^4 or 10^3 cells showed a decrease in incidence of tumour takes from VCN-treated inocula compared with the corresponding controls. The combined figures for tumour incidence at these three dose levels show a total of 13/37 (35 ± 2.8%) for VCN treated cells, which

Table 1. Sialic acid release (nmole/10^9 cells) *during treatment with low and high concentrations of VCN by procedures (A) and (B) (Mean values ± S.E.M.)*

	Procedure (A)		Procedure (B)	
	Control	VCN (25 U/ml, pH 7.4)	Control	VCN (500 U/ml, pH 5.5)
Sialic acid in supernatants from				
incubation	33 ± 9 (8)	239 ± 21 (8)	77 ± 8 (5)	196 ± 10 (3)
washings			58 ± 8 (5)	36 (2)
Total free sialic acid released	33	239	135	232
As % of total sialic acid in cells	6%	50%	28%	49%

is significantly lower ($P=0.02$) than the control value of 22/35 ($61 \pm 6.6\%$) for the controls. An additional phenomenon observed in Schofield mice was a significant ($P<0.01$) reduction in 15-day tumour yield in mice inoculated with 10^5 VCN-treated cells (Table 2). It can be seen that more concentrated inocula, from 10^6 up to and including 2.5×10^7, resulted in no difference between VCN-treated and control groups in incidence of takes or weights of tumour yield. (1.5×10^7–3.0×10^5 cells/mouse) but a significant reduction in tumour weight was observed in the group receiving 4×10^6 VCN-treated cells.

The data in Table 2 show that prior exposure of the cells to 25 U VCN/10^7 cells/ml at pH 7.4 affected tumour transplantability to a distinct degree in 2 of the 3 mouse strains tested provided that the number of cells administered was below the tumour ED_{100} for that strain, i.e. the

Table 2. Transplantability in three mouse strains after exposure of tumour cells to VCN (25 U/10^7 cells/ml) *according to procedure (A)*

		Control cells		VCN-treated cells	
No. of cells injected/ mouse	Growth period (days)	Incidence* of takes	Tumour dry† wt. mg/mouse (mean ± S.E.M.)	Incidence* of takes	Tumour dry† wt. mg/mouse (mean ± S.E.M.)
Schofield					
2.5×10^7	7	10/10	196 ± 22 (10)	10/10	209 ± 19 (10)
5.0×10^6	7	5/5	137 ± 36 (5)	5/5	175 ± 12 (5)
2.5×10^6	7	6/6	111 ± 25 (6)	6/6	100 ± 18 (6)
1.3×10^6	7	6/6	69 ± 8 (6)	6/6	54 ± 25 (6)
1.0×10^6	14	4/4	372 ± 22 (4)	4/4	282 ± 45 (4)
1.0×10^5					
(a)	14	3/5	264 ± 37 (3)	0/7	nil (7)
(b)	14	8/10	172 ± 21 (8)	6/10	72 ± 20 (6)‡
1.0×10^4	14	5/10	34 ± 10 (5)	4/10	43 ± 13 (4)
1.0×10^3	14	6/10	31 ± 5 (6)	3/10	21 ± 2 (3)
1.0×10^2	14	0/10	nil (10)	0/10	nil (10)
BDF/1					
2.4×10^7	7	4/5	128 ± 33 (4)	6/6	165 ± 20 (6)
6.0×10^6	8	8/8	165 ± 14 (8)	8/8	144 ± 10 (8)
1.0×10^6					
(a)	16	5/7	328 ± 52 (5)	1/7	157 (1)
(b)	14	4/5	178 ± 92 (4)	0/6	nil (6)
6.0×10^5	18	1/6	363 (1)	1/6	544 (1)
A_2G					
1.5×10^7	7	5/5	114 ± 7 (5)	6/6	108 ± 13 (6)
4.0×10^6	14	5/5	380 ± 48 (5)	5/5	206 ± 39 (4)§
1.0×10^6	15	6/6	389 ± 26 (6)	6/6	311 ± 24 (5)
5×10^5	14	5/5	259 ± 12 (4)	5/5	229 ± 64 (5)
3×10^5	16	7/7	259 ± 33 (7)	6/7	341 ± 47 (6)

*Number of mice which developed tumour/number injected.
†Calculated per mouse in which tumour developed.
Significant reductions in mean tumour yield denoted by ‡ ($P<0.01$) and § ($0.02<P<0.05$).

Similarly, in BDF/1 mice, VCN-treated cells developed tumour to the same extent as control-treated cells when the upper inoculum levels (6×10^6–2.4×10^7 in this case) were used, but showed almost complete inhibition of development when administered in a dose of 1×10^6/mouse. In addition, the one BDF/1 mouse that developed tumour from 1×10^6 VCN-treated cells showed a reduced tumour yield. In A2G hosts VCN treatment had no effect on the incidence of takes over the range of inocula used inoculum level required to produce 100% tumour takes in the control group under our conditions.

In addition to short-term assessments, long-term survival was also tested in mice which had been given similarly treated cells. Two groups of Schofield mice were injected with 4×10^6 cells each, control or VCN-treated. The same dose was also given to 17 BDF/1 mice (8 control, 9 VCN) and all animals were observed daily. All mice in untreated and treated groups of each

strain were moribund from tumour by 30 days and were therefore killed. In a further experiment 2 groups of 10 Schofield mice received 10^2 cells, treated or untreated. Due to the low cell dose, neither group showed any obvious signs of tumour at the end of 2 weeks (cf. Table 2); nevertheless all eventually became equally distended with tumour and were therefore killed at 7 weeks. These survival experiments confirmed the results of short-term tests in showing that VCN-treated cells develop normally *in vivo* when administered in doses outside the critical ranges demonstrated by the results in Table 2.

Cells treated according to procedure (B). Currie and Bagshawe [6] reported that only 1/40 A2G mice injected with 4×10^6 VCN-treated Landschütz cells died from tumour, compared with a death rate of 100% in the control group. Since we had found no marked inhibition of transplantability in this mouse strain at any of the inoculum levels tested, we carried out additional experiments in which the treatment conditions used by these authors were closely followed. The cells were then used to test the transplantability in A2G mice of inocula of 4×10^6 and 2.5×10^5 cells.

It has already been shown (Table 1) that no extra free sialic acid was removed from the cells by using this greatly increased VCN concentration. The results of *in vivo* tests were as follows: 14 days after an inoculum of 4×10^6 cells the control group showed 6/6 tumour takes and a mean tumour yield of 234 ± 57 mg, the corresponding values for the VCN-treated group being 5/6 takes and 402 ± 32 mg/mouse. Thus the reduction in tumour yield found in A2G mice injected with 4×10^6 cells treated with VCN according to procedure (A) was not observed when procedure (B) was used to treat the cells. A further 7 mice in the treated group were observed for 25 days after injection, at which stage 2 were tumour-free. The remaining 5 (71%) were obviously near death and when killed yielded a mean wt of 388 ± 30 mg/mouse. On reducing the inoculum to 2.5×10^5, tumour takes after 21 days were 2/5 in both the control and the VCN-treated group and the mean tumour yields were 260 mg (2) and 158 mg (2), respectively.

Thus, VCN treatment under the conditions outlined in procedure (B) permitted normal tumour development in 66% (12/18) of A2G mice, compared with 72% (8/11) in the relevant controls.

Cytotoxicity of host sera

When cells subjected to procedures (A) or (B) were incubated with sera from host mice as described in Methods, viability was found to be unaltered in both control and VCN-treated samples. Thus, non-tumour-bearing Schofield, BDF/1 and A2G mice do not appear to carry serum factors directly cytotoxic to either VCN-treated or control-treated Landschütz cells.

DISCUSSION

In testing for an effect of VCN on tumour transplantability valid conclusions can only be drawn if the viability of the enzyme-treated inoculum can be shown to be normal. The report of Yuhas *et al.* [15] described a VCN-induced cytotoxic effect on line-1 cells which was not detectable by dye exclusion methods. In the present study, however, we found tumour development from VCN-treated cells to be normal in all 3 host strains when the upper ranges of inoculum levels were used. This indicates that VCN-treated and control cells were of equal viability and thus provides confirmation of our results using vital dyes.

It was interesting to find (Table 1) that free sialic acid was removed from the cells by VCN to the same extent using treatment procedures (A) and (B). This suggests that the amount concerned, 50% of the total content in these cells, is the maximum removable by VCN exposure and that this can be achieved using 25 U/ml at pH 7.4. Similarly, Bekesi *et al.* [8] found 20–50 U/ml to be optimal for the ascites tumour L 1210. Currie [5], although not measuring sialic acid release, found a progressive decrease in electrophoretic mobility with doubly increasing doses of VCN up to and including 500 U/ml. Our finding that no extra free sialic acid is removed by the use of high enzyme concentrations provides additional evidence for the proposal [1, 18] that the use of excessive VCN can decrease electrophoretic mobility by means other than removal of sialic acid, e.g. through absorbance of excess enzyme on the cell surfaces.

Our use of 500 U/ml was motivated by the work of previous authors [5, 6] who, under these conditions, found almost total inhibition of transplantability in A2G mice and showed [5] this concentration to be essential for the effect. Our present results, however, do not show any marked decrease in transplantability in these mice. The reason for this divergence is not clear.

As already mentioned, the results in Table 1 show a marked difference between the two controls in procedures (A) and (B) in that considerable loss of free sialic acid occurred in control cells subjected to procedure (B), amounting to 28% of total cell content. Theoretically therefore, it might be expected that these cells would be less oncogenic than

control cells prepared by procedure (A), but our results indicate that this is not the case: A2G mice showed 100% takes from 4×10^6 cells prepared by either procedure and the mean 14-day tumour yields/mouse of 380 ± 48 (5) and 284 ± 57 (6) (procedures (A) and (B) respectively) do not differ significantly. This suggests that a greater proportion than 28% of the total sialic acid has to be removed from Landschütz cells before tumourigenicity is reduced. It has been shown for L 1210 ascites tumour [8] that maximum enzymatic removal of free sialic acid must be achieved before an effect on transplantability is manifested.

The main point arising from the present study is that the choice of inoculum level can be of major importance for detection of an effect of VCN on transplantability, since at or above the ED_{100}, as defined above, no effect on tumour incidence was observed. That VCN can strongly reduce the transplantability of Landschütz ascites tumour in 2 of the 3 mouse strains tested is evident from the results using certain inoculum levels below the ED_{100} (Table 2). This finding may be compared with earlier observations [19] that large inocula of chemically transformed cells were able to override the immune system of host mice and grow progressively but medium sized inocula were rejected. The tumour doses at which an effect of VCN was observed were 1×10^6 cells for BDF/1 and between 1×10^5 and 1×10^3 cells for Schofield mice. In A2G hosts VCN treatment by either procedure had no effect on the incidence of tumour takes.

An additional phenomenon, found in Schofield mice, was a significant reduction in the 15-day yield of tumour weight in mice which had received 1×10^5 VCN-treated cells. This effect appeared to be specific for an inoculum of 10^5 cells. The results were repeatable in two experiments separated by a period of 1 yr and were not due to variations in experimental technique, since mice receiving higher and lower cell numbers were inoculated using the same concentrated incubation mixtures. A similar trend is seen in BDF/1 mice given 1×10^6 cells and in A2G mice receiving 4×10^6 cells.

It has been suggested [20, 21] that the transplantability of VCN-treated cells may depend on the absence or presence of antibody-like cytotoxic factors pre-existing in the sera of some host animals which can lyse VCN-treated but not untreated tumour cells. The *in vitro* cytotoxicity described by these authors was, however, dependent on the addition of guinea-pig complement (despite the fact that the mouse sera had not been heat-deactivated) and therefore would not seem to have any bearing on the *in vivo* fate of VCN-treated cells in mice. Furthermore, while VCN increases the sensitivity towards added complement of lymphoid cells [22] and Landschütz tumour [23], the latter author also found a similar effect after exposure of Landschütz cells *in vitro* to the proteolytic enzyme brinase, which has a growth enhancing effect on this tumour in Schofield mice [24]. We therefore considered direct single-stage cytotoxicity tests to be more relevant to the present problem. These were performed according to the method of Sethi and Brandis [9], who have described preferential lysis of VCN-treated Ehrlich and L 1210 ascites tumour cells *in vitro* by fresh serum from C3H, DBA, C57 and NMRI mice but not from BALB/c strain. We found no evidence of cytotoxicity in the serum of Schofield, BDF/1 or A2G mice towards control or VCN-treated cells which had been subjected to procedures (A) or (B). It appears, therefore, that in the mouse strains used in this study, VCN-induced inhibition of tumour was not mediated by pre-existing serum cytotoxic factors.

Sanford and Codington found that VCN reduced the transplantability of TA3 ascites tumour cells in C3H mice but permitted them to grow normally in DBA and A mice [21]. They attributed this to the existence, in C3H serum but not in that from the other two strains, of a cytotoxic activity in the presence of added complement, which was preferentially active aginst VCN-treated TA3 cells. We suggest, however, that their findings can be readily interpreted in terms of our present results, which emphasise the importance of using the optimum inoculum level for transplantability effects in each host strain. For example, their results (Table 1) show that in DBA and A mice the inoculum used (3×10^3 cells) caused the death of 100% of the control group and therefore was equal to, or possibly greater than the LD_{100} (or ED_{100} as defined earlier for our conditions) of the tumour in these hosts; hence, administration of the same number of VCN-treated cells had no effect on the incidence of tumour takes. In C3H mice, in contrast, the inoculum of 3×10^3 cells was only 75% effective in the control group and here prior treatment with VCN resulted in a reduction of approximately 50% in tumour incidence. Hauschka *et al.* [12] also used TA3 tumour in C3H and A strain mice and found (their Table 6) that 3×10^3 cells injected into A mice resulted in 100% tumour takes in the control group and the same effect when VCN was used. An inoculum of 3×10^3 cells from a less than normally viable suspension was evidently below the LD_{100} for strain A mice, since only 87% of the control group developed tumour and

in this case a slight reduction due to VCN (70%) was seen. Again, in C3H mice administration of fully viable cells only resulted in 78% takes in the controls and some effect of VCN on transplantability was evident, the incidence of tumour development falling to 63%. The use of a less than normally viable inoculum in C3H mice resulted in only 10% tumour takes from 3×10^3 untreated cells and no reduction in transplantability in the VCN-treated group: the low incidence of takes in this instance indicated that the tumour dose is outside the optimal range for detection of an effect of VCN as evidenced by the results in Table 2 of the present study.

It is suggested that other instances may exist where failure to observe reduced transplantability of VCN-treated tumour cells could be attributable to the use of unsuitably high tumour inoculum levels. Further work using a range of tumour-host systems may help to explain some of the divergencies in the literature on this subject.

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Lysosomal Enzyme Inhibitors and Antimetastatic Activity in the Mouse*

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Abstract—*Saccharo-1,4-lactone, a β-glucuronidase inhibitor, and three protease inhibitors did not affect primary tumour growth when tested against Lewis lung carcinoma in mice. The number of lung metastases was significantly reduced by aprotinin only. The inactivity of leupeptin and pepstatin seems to indicate that cathepsin B and D are not involved in metastasis formation. These results also indicate that aprotinin, a broad spectrum protease inhibitor, reduces tumour dissemination, in addition to the tumour invasion already reported by others.*

INTRODUCTION

THE ROLE of lysosomal enzymes in such malignant properties of solid tumours as invasiveness and formation of metastases has received the attention of several investigators. Although some controversy concerning the methodology of the assays arose in one instance [1], it is generally accepted that certain enzymes such as cathepsins, collagenase, hyaluronidase and β-glucuronidase, present in higher concentrations in tumour cells, tumour cell surface and/or the interstitial fluid of solid tumours than in normal tissues, might be responsible for tumour invasion and dissemination [2–4].

This evidence is further supported by the reported effects of enzyme inhibitors on *in vivo* tumour growth. The treatment of mice bearing Ehrlich carcinoma with saccharo-1,4-lactone, a β-glucuronidase inhibitor, markedly reduced tumour growth [5]. Aprotinin, a broad spectrum protease inhibitor, reduced tumour growth and invasion as well as increasing tumour necrosis, when administered to hamsters bearing a highly invasive fibrosarcoma or to mice bearing a similarly malignant mammary carcinoma [6]. The survival time of mice inoculated with a malignant thymoma was increased by the peritumoural administration of cysteine, an inhibitor of collagenase [7].

N-diazoacetyl glycine amide (DGA), a substance which belongs to a class of active site directed, irreversible inhibitors of proteolytic enzymes [8], has recently been shown to possess antimetastatic properties in mice bearing Lewis lung carcinoma [9], in addition to its previously reported antitumour effects on rodent transplantable neoplasms [10].

These reports and considerations led us to use the same experimental system to test the possible antimetastatic effects of a series of lysosomal enzyme inhibitors.

MATERIAL AND METHODS

The animals used for this investigation were C57Bl/6 Cr male mice, weighing 21–22 g. Tumour transplantation was performed by trocar s.c. implantation in the axillary region of a tumour homogenate, prepared using donor mice similarly inoculated 2 weeks before [11]. Primary tumour weight was evaluated by caliper measurement of tumour diameters [12]. Lung colonies were detected using the technique described by Wexler [13].

Aprotinin and saccharo-1,4-lactone were obtained from commercial sources (Bayer and Sigma respectively). Pepstatin and leupeptin were generously provided by Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan.

The substances were administered i.p. as an aqueous solution using 0.1 ml (0.2 ml for aprotinin) per 10 g of animal weight.

RESULTS AND DISCUSSION

The experimental results obtained are reported in Table 1. For all the tested compounds, the effects on primary tumour growth were negligible and not statistically significant.

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Table 1. Effects of lysosomal enzyme inhibitors on Lewis lung carcinoma

Treatment		Effects on primary tumour		% Body weight variation compared with controls at the end of treatment	Effects on metastases	
Drug	Daily dose/kg	Average tumour weight g (±S.E.)	T/C*		Average number (±S.E.)	T/C*
Controls		3.62±0.51			15.0±2.5	
Aprotinin	200.000 KIU	2.53±0.55	0.70	−0.8	7.62±3.3†	0.51
Pepstatin	50 mg	3.26±0.48	0.90	+0.8	20.8±3.2	1.39
Leupeptin	50 mg	2.91±0.46	0.80	−7.7	16.9±1.7	1.13
Saccharo-1, 4-lactone	150 mg	2.82±0.46	0.78	−0.6	17.0±2.0	1.13

*T/C is the ratio of the average values of each treated group to that in controls. The treatment was performed daily, from day 1 to 14 after tumour inoculation: groups of 8 mice were used. The primary tumour mass was measured on day 15, and the lungs examined for metastases at sacrifice on day 24. The statistical analysis performed is a one way analysis of variance [21].

†Mean significantly different, $P < 0.05$.

At a dosage which has been reported to markedly inhibit *in vivo* tumour growth [5], saccharo-1,4-lactone was devoid of any effect on the number of metastases found at sacrifice. Among the protease inhibitors tested, pepstatin and leupeptin were similarly inactive, whereas aprotinin significantly reduced the number of lung colonies to about 50% of controls.

The possibility that the inactivity observed for leupeptin and pepstatin in our experimental system may be due to insufficient dosage can be ruled out on the basis of the following considerations. The leupeptin dose used has been shown to inhibit the *in vivo* carcinogenic effects of polycyclic hydrocarbons and also reduced the TAME esterase activity in the skin of the treated mice [14]. Pepstatin was administered at the same dosage used for leupeptin, and its effectiveness in inhibiting cathepsin D *in vitro* is 40 times greater than that of leupeptin for cathepsin B [15].

In addition to the reported reduction of the malignant growth of rodent tumours [6], these findings indicate that treatment with the protease inhibitor, aprotinin, also causes a depression in the number of haematogenous metastases in the experimental model employed. They are in agreement with the reported reduction of macroscopic metastases caused by aprotinin in hamsters bearing a fibrosarcoma, which was attributed to the enhancement of the immunological system of the host against the tumour cells [16]. In contrast to previous reports concerning the role of cathepsins, in particular cathepsin B, in the process of metastasis formation [17], the observed lack of activity of leupeptin and pepstatin seems to rule out the participation of cathepsin B and D in this process. These data also support the hypothesis that the antimetastatic effects reported for DGA might be attributed, at least in part, to an inhibition of proteolytic enzymes. Diazoacetyl derivatives of amino acids have been shown to inhibit acidic proteases via a k_{CAT} mechanism [18]; the inhibition being more pronounced in the presence of divalent metallic cations [19, 20]. Although the mechanism has still to be elucidated, these compounds have also been found to inhibit neutral proteases in the absence of cupric ions [22].

Further work is in progress aimed at testing additional inhibitors, of natural and synthetic origin and to see whether their mechanism of antimetastatic action *in vivo* involves actual inhibition of tumour proteases. The purpose of this investigation is to increase the knowledge of the process of metastasis formation and to increase the number of agents different from conventional cytotoxic drugs available for cancer treatment.

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Histochemical, Light and Electron Microscopic Study of Polonium-210 Induced Peripheral Tumors in Hamster Lungs: Evidence Implicating the Clara Cell as the Cell of Origin*

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Abstract—*Peripheral lung tumors induced in Syrian golden hamsters by intratracheally administered polonium-210 (^{210}Po) are similar to the peripheral lung tumors induced in many species by a variety of carcinogens. In addition, they show many of the histopathological features observed in human bronchiolar–alveolar carcinomas. Serial sacrifice studies of hamsters exposed to multiple instillations of ^{210}Po have been carried out to identify the cell of origin of these tumors. By means of thin, plastic (glycol methacrylate) sections, electron microscopy, and histochemistry, we conclude that the bronchiolar Clara cell is the probable cell of origin, and that this view is generally compatible with many of the reported cytological characteristics of the human tumor.*

INTRODUCTION

INTRATRACHEALLY administered polonium-210 (^{210}Po) is a potent lung carcinogen in Syrian golden hamsters, with tumor induction time and incidence related to dose over a broad range [1]. The tumors arise in the bronchiolar–alveolar region, and because they may show light microscopic features of both epidermoid and adenocarcinomas, have been classified by us as "combined tumors" [1]. The tumors themselves, as well as the precursor lesions, are often associated with a variable degree of periodic acid–Schiff (PAS)–positive secretory product.

^{210}Po is an alpha emitting radioisotope and is present in cigarette smoke [2] and cigarette smokers' lungs [3]. ^{210}Po has been shown to be carcinogenic to hamsters at whole lung doses as low as 15 rad [4]. In man, bronchiolo-alveolar carcinoma is a peripheral lung tumor similar in appearance to the ^{210}Po induced tumors; it is estimated to represent as much as 8% of all lung tumors [5] and there is some evidence that smoking plays a significant role in its pathogenesis [6].

Human bronchiolo-alveolar carcinoma, also known as alveolar cell cancer, malignant pulmonary adenomatosis, bronchiolar carcinoma etc. [7], is thought to arise from bronchiolar or alveolar epithelium. The tumor was first described by Malassez [8], who characterized the multiple nodular, and therefore possibly multicentric, form of the disease. However, Storey [9] in 1953 showed it to be of unicentric origin and thus a likely possibility for a surgical cure. Bronchiolar–alveolar carcinoma exhibits two basic cellular forms [9–12]. The first is a well differentiated carcinoma with a uniform, tall columnar cellular pattern and abundant mucin production. Mucin is present within the cytoplasm of tumor cells as well as in extracellular spaces. Since the tumor cells tend to grow along, rather than invade, the alveolar walls, the basic bronchiolar–alveolar architecture is maintained. Ciliated cells are rarely found, and there are rather few mitotic figures. The second form is less well differentiated and characterized by more solid areas of tumor with concomitant loss of alveolar architecture. The tumor cells are larger, pleomorphic and hyperchromatic [9];

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mucin production is infrequent or absent. In either form of the tumor, one may find areas of epidermoid differentiation [10]. According to McNamara *et al.* [11], the extent of cellular maturation is not related to the aggressiveness of these tumors and, as a group, the 5 yr survival is much higher than for epidermoid or adenocarcinomas of bronchogenic origin.

Experimentally induced ^{210}Po tumors also show considerable morphologic variations: among animals, among lobes in the same animal, and within microscopic fields of a large lesion; in the degree of glandular differentiation; in the degree of epidermoid differentiation or metaplasia; and in the presence of mucin. Even when the tumor cells appear cytologically innocent, some of these induced tumors show clear evidence of invasive and metastatic spread.

The cell of origin of the human tumor is controversial. There is some evidence that the tumor cells have ultrastructural characteristics of bronchiolar epithelium [13, 14]; however, several recent studies have attributed the origin to neoplastic type II alveolar epithelial cells [12, 15]. Type II alveolar epithelial cells have also been implicated as the cell of origin of many animal tumors of the peripheral lung [16–18], particularly mouse adenomas [16, 17]; however, the histogenesis of mouse adenomas may be very different from peripheral lung tumors of other species.

The cell of origin of the ^{210}Po induced tumors was not clear from our earlier work with animals bearing end-stage tumors [1]. The present series of experiments were designed to study the natural history of those lesions in the lungs and to determine the cell of origin. Three approaches have been used for this study: (1) a serial sacrifice experiment utilizing one-micron sections of glycol methacrylate (plastic) embedded lungs to study the early lesions in the lungs; (2) electron microscope sections of induced tumors to determine the ultrastructural characteristics of the cells involved; and (3) histochemical studies of tumors to determine the nature of the tumor secretions.

Identification of the cell type originating peripheral lung tumors will enable the calculation of radiation doses necessary to induce malignant transformation in that sensitive cell type. The relative hazards of various lung carcinogens can then be determined in terms of doses to critical cells. An estimation of risk from the inhaled plutonium isotopes, having alpha particle emissions similar to the ^{210}Po alpha particle, is particularly important due to the increasing likelihood of human exposure to these alpha emitting isotopes produced by the nuclear power industry. Plutonium isotopes have been shown to produce primarily bronchiolar–alveolar carcinomas in many species under a variety of exposure conditions [19]; these peripheral lung tumors are histologically like those seen in man [7–12] and our ^{210}Po exposed animals [1].

MATERIAL AND METHODS

Random-bred, male Syrian golden hamsters (Dennen Animal Industries in Gloucester, Mass.) weighing 100–125 g., were given 7 intratracheal instillations of 0.1 μCi ^{210}Po in 0.9% NaCl as has been previously described [1]. Animals were sacrificed weekly during and after the instillations for 60 weeks. All animals were sacrificed by Sodium Brevital (Lilly) overdose and exsanguination from a renal artery. Preparation of lungs for 1 μ glycol methacrylate (plastic) sections and staining procedures have been described in detail [20]. Tissue samples were cut from each lobe in planes parallel to the axis of the major bronchus, and from areas of suspected pathology.

For electron microscopy, three animals were sacrificed at 60 weeks after the instillation period and lungs were prepared for examination as follows. Lungs were inflated in the chest by cannulation (19-gauge needle, PE 190 Intramedic tubing) and injection of Karnovsky's glutaraldehyde-paraformaldehyde fixative [21] at 4°C (30 cm water pressure). After 5 min fixation in the chest, lungs were removed and placed in a beaker of Karnovsky's fixative for 3 hr. Lungs were cut into small pieces (less than 1 mm square), washed 4 times in cacodylate buffer (0.1 M, pH 7.4) and stored overnight in cold buffer. The blocks were then post-fixed in s-collidine-buffered osmium tetroxide (1.3% osmium tetroxide in 0.1 M s-collidine, pH 7.4) for 1 hr, washed 4 times in s-collidine buffer, dehydrated and embedded in epon. Tissue sections were cut on a Reichert ultramicrotome. One micron sections were stained with toluidine blue or PAS-hematoxylin. Appropriate blocks were trimmed for electron microscopy, and silver sections cut with a diamond knife (DuPont). Sections were mounted on 300 mesh copper grids, and stained with uranyl acetate and lead citrate. Sections were examined with a Philips 300 electron microscope.

To determine the nature of the secretion produced by the tumor cells, by the cells in non-neoplastic areas, and in control lungs, various histochemical staining procedures were used on sections from animals sacrificed 60 weeks after the instillation period and from normal controls.

For those stains in which chemical processing techniques would interfere with the histochemical nature of the tissue, frozen sections were used. For these studies, the collapsed lungs and trachea were removed *en bloc*, the trachea cannulated with PE 190 polyethylene tubing, lungs filled with O.C.T. embedding medium (Fisher Scientific Co.) and frozen rapidly by placing them for 1 min in isopentane cooled to the viscous stage by liquid nitrogen. The frozen lungs and trachea were then cut into pieces, mounted on chucks, and cut at 4 μ in a microtome cryostat (Harris Mfg. Co.) maintained at $-35°C$. For some of the stains used in this study, paraffin embedded tissue was commonly utilized. For paraffin sections, the inflated lungs and trachea were fixed in 10% neutral buffered formalin and embedded in paraffin blocks using conventional procedures.

The following staining procedures were done on frozen sections:

Sudan black B—formalin fixed frozen sections with acetone extraction [22];

PAS—with and without diastase digestion [23];

Best's carmine method for glycogen [23];

Hale's colloidal iron method [23]; and

Baker's acid hematein method—with and without pyridine extraction [23].

The following stains were done on paraffin sections:

PAS—with and without prolonged acetylation [23];

Mayer's mucicarmine method for mucin [24];

Muller's modification of Mowry's colloidal iron stain for acid mucopolysaccharides with Van Gieson's collagen fiber stain—with and without sialidase digestion [24];

Alcian blue pH 0.4 [24];

Alcian blue pH 2.5—PAS sequence [24];

Alcian blue pH 1.0—PAS sequence [24]; and the high iron method [25].

RESULTS

I. Serial sacrifice study, 1 μ sections—glycol methacrylate embedded lungs. Since a number of histopathologic findings in this study have been described elsewhere [26], only those results pertinent to the cell of origin of these tumors will be reported here.

As early as 7 days after the second instillation of ^{210}Po, type II alveolar epithelial cells responded to the treatment by undergoing hyperplasia and hypertrophy with an increased number of cytoplasmic inclusions or cytosomes. Subsequently, these enlarged cells were also often seen within tumors and in groups surrounding tumor areas. At 7 days after the 5th instillation of ^{210}Po, the first example of "alveolar epithelialization or bronchiolization" was seen (example, Fig. 1). By 4 weeks following the last instillation of ^{210}Po, such foci were seen in almost all animals sacrificed. Briefly, bronchiolar epithelial cells are found lining the surface of preexisting alveoli, where normally only alveolar-type cells occur. Both ciliated and typical bronchiolar columnar and cuboidal cells appear in these hyperplastic nodules [26], although ciliated cells were less frequently seen as the pathologic regions containing acinar structures became larger in size. These hyperplastic nodules were often seen with alveolar macrophages trapped inside. As early as 10 weeks after the last instillation of ^{210}Po, they consisted of many cells resembling Clara cells but containing PAS positive secreted materials (Fig. 2). Both single- and double-layered hyperplastic nodules were found. It is of interest that many Clara cells of the normal bronchiolar epithelium, not normally PAS positive cells, also began to display PAS positive granules (Fig. 3) similar to those seen in cells of the hyperplastic and tumor regions (Figs. 4 and 5). Squamous metaplasia was seen in many of the acinar structures (Fig. 6).

True lung tumors (combined epidermoid and adenocarcinomas), which we believe arise in these hyperplastic nodules, are aggressive, epithelial lesions which are usually multicentric, show varying cellular atypia, destroy the supporting lung stroma, and which occasionally invade airways, blood vessels or pleura. Mucus production is nearly always present. These tumors are transplantable [27], and their histopathologic characteristics have been described in detail elsewhere [1].

II. Electron microscopic study. One-micron epon sections from the three animals studied by light microscopy showed the presence of ^{210}Po induced tumors of the type previously described [1]. In addition, lung fields adjacent to the neoplasms and in other lobes showed many areas of alveolar epithelialization and hyperplastic nodules. Some of these foci were not associated with a secretory product (Fig. 1). Electron microscopic examination of these hyperplastic nodules showed cells resembling Clara cells but containing many more light secretion granules than usually found in normal hamster Clara cells [28].

The following cell types were found within the tumors:

A. The most frequent cell type encountered within the acinar structures was a cell covered by

prominent microvilli supported by tonofilaments, and having nuclei of irregular contour, often bilobed (Figs. 7 and 8) and containing clumped chromatin around the periphery (Fig. 7). These cells have a well-developed Golgi zone (Fig. 7), extensive granular endoplasmic reticulum (Figs. 7 and 8), often much apical agranular endoplasmic reticulum (Fig. 8), and many free ribosomes (Figs. 7 and 8). Membrane limited, electron dense mucous granules could be seen in the apical cytoplasm. Mitochondria were numerous, often swollen, and contained irregular cristae which only partially traversed the mitochrondria (Figs. 7 and 8). Lysosomes were often seen, and there were frequent myelin figures (Fig. 8). There were "dark" and "light" cells—the electron density depending on the amount of free ribosomes and agranular endoplasmic reticulum. Cell to cell connections included terminal bars and desmosomes.

B. Cells with clear evidence of epidermoid differentiation having irregular nuclei, tonofibrils (Fig. 9), bundles of tonofibrils, numerous desmosomes (Fig. 9), complex interdigitations between cells (Fig. 9), microvilli, vesiculation of the endoplasmic reticulum, many free ribosomes and some agranular endoplasmic reticulum. There are "dark" and "light" cells in this category, depending mainly on the amount of agranular endoplasmic reticulum present. Small round intracytoplasmic inclusion bodies, with a dense core of variable composition, were often seen in these cells (Figs. 9a and b). These particles were all smaller than 0.25 μm.

C. Another prominent cell type seen in the acinar structures, as well as in areas where no such structures were visible, was a cell resembling the Clara cell with prominent microvilli, extensive supranuclear agranular endoplasmic reticulum, nuclei having condensed chromatin around the edges, and many membrane bound granules of electron lucent material (Fig. 10). There were numerous small round or oblong mitochondria and occasional lysosomes. Myelin figures were often present.

D. Undifferentiated cells consisting primarily of ground substance with microvilli, tonofilaments, very deeply infolded nuclear membranes, some granular endoplasmic reticulum and particulate glycogen (Fig. 10).

E. Ciliated cells (Fig. 10)—having numerous apical mitochondria, a large Golgi zone, many ribosomes and lysosomes, many cilia, occasional centrioles and some microvilli. Most of these cells appeared to be regular ciliated cells, as have been described at the electron microscopic level in detail elsewhere [29].

F. Enlarged type II alveolar epithelial cells (Fig. 11)—very similar to normal type II alveolar epithelial cells, described elsewhere [29]. They were considerably larger than normal, with many more cytosomes or lamellar bodies than are usually found. With our system of processing, the cytosomes are extracted to a large degree. In the cytoplasm were numerous mitochondria, ribosomes, some granular endoplasmic reticulum, and a few lysosomes. There were occasional microvilli and large round nuclei.

There were also occasional cell types with extensive development of the endoplasmic reticulum or Golgi apparatus, or with very long microvilli. Other cell types found in the tumor, but probably having little to do with the malignant state, include macrophages (Fig. 10), polymorphonuclear leukocytes, connective tissue cells and blood and blood vessel cells, all of which have been described at the electron microscopic level elsewhere [29].

III. Results of histochemical studies for characterization of the secretory products, both in apical cytoplasm of tumor cells and extracellularly, in hyperplastic nodules and in adjacent Clara cells in bronchioles. Glycogen stains were negative. All of the mucin stains were positive in areas; the results have been interpreted as follows. The PAS positive granules and secretions within the acinar structures of tumors have the staining characteristics of both acidic and neutral mucosubstances. Although occasional sulfomucins (acidic mucosubstances) were observed, the primary acidic product is a sialomucin, neuraminidase resistant, a product also seen in some atypical Clara cells in bronchioles near tumor areas, as well as in some goblet cells and tracheobronchial glands. Most of the atypical Clara cells in bronchioles secrete a neutral mucopolysaccharide, a product also associated with hyperplastic nodules, secretory focal lesions with squamous metaplastic features, and many of the cells in acinar structures within the tumors themselves. Sudan black staining of frozen sections showed some sudanophilic areas not removed by acetone extraction, and as shown with the Baker acid hematein stain, most likely a phospholipid. A few of the atypical cells within bronchioles near tumor regions were also Sudan black B positive (with acetone extraction); however, most Clara cells were not sudanophilic.

DISCUSSION

What we regard as the first stage in the spectrum of responses leading to tumors is what is known as "epithelialization or bronchioliz-

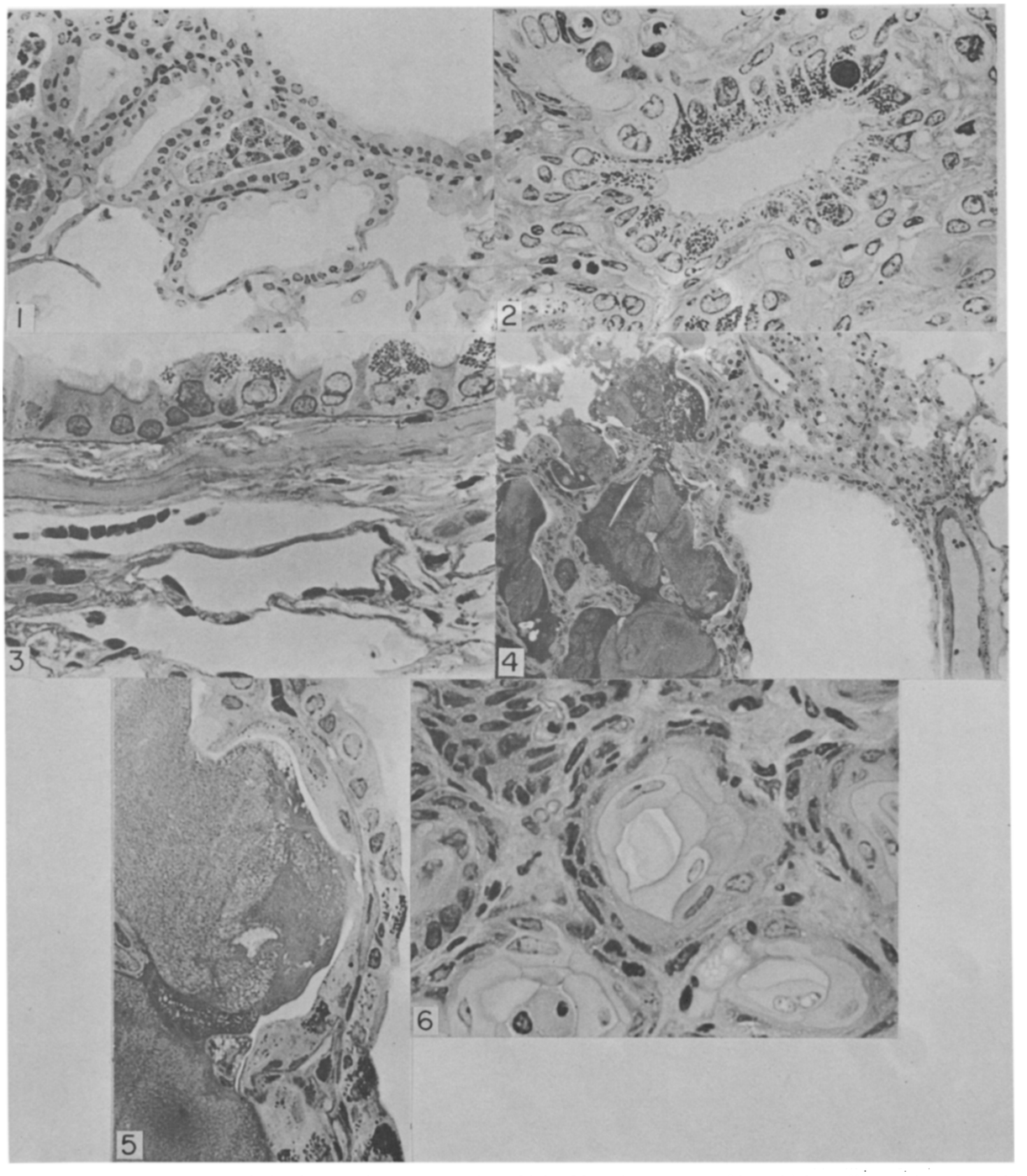

Fig. 1. Epithelialization of alveoli occurring next to a bronchiole. Note the presence of bronchiolar cells growing along the alveolar walls in a glandular pattern. Most of the epithelial cells are typical Clara cells, but ciliated cells are also found. Macrophages (center) are often seen trapped within the acinar structures. Four weeks after the ^{210}Po instillation period. PAS-hematoxylin. × 250.

Fig. 2. A single cell layered hyperplastic nodule within a ^{210}Po induced tumor at an early stage of development. Mucus can be seen as PAS positive granules in the apical region of cells within the acinar structure (center). Note that the nuclei of these cells are characteristic of Clara cells, often bilobed and containing condensed chromatin around the edges. Twenty-seven weeks after the ^{210}Po instillation period. PAS-hematoxylin. × 500.

Fig. 3. Bronchiole (top) near a tumor region of this lung. Normal bronchiolar Clara cells do not have a PAS positive secretion, but Clara cells exposed to ^{210}Po begin to secrete PAS positive mucous granules (top center, right). 18 weeks after the ^{210}Po instillation period. PAS-hematoxylin. × 500.

Fig. 4. Relatively normal bronchiole (right) and tumor region (left). There are normal Clara cells on the right side of the bronchiole and atypical Clara cells (mucus-secreting) on the left side. An artery appears at the right of the bronchiole. Twenty-nine weeks after the ^{210}Po instillation period, PAS-hematoxylin. × 125.

Fig. 5. Higher power view of left side of bronchiole shown in Fig. 4. Bronchiolar epithelium (far right) contains typical Clara cells (top) and atypical Clara cells (bottom) with PAS positive secretion granules. Note similarity of atypical bronchiolar cells to the epithelial tumor cells growing along the alveolar wall adjacent to the bronchiole. Twenty-nine weeks after the ^{210}Po instillation period. PAS-hematoxylin. × 500.

Fig. 6. Area of squamous metaplasia occurring within a ^{210}Po induced tumor. Hematoxylin-phloxine. Nineteen weeks after the ^{210}Po instillation period. × 500.

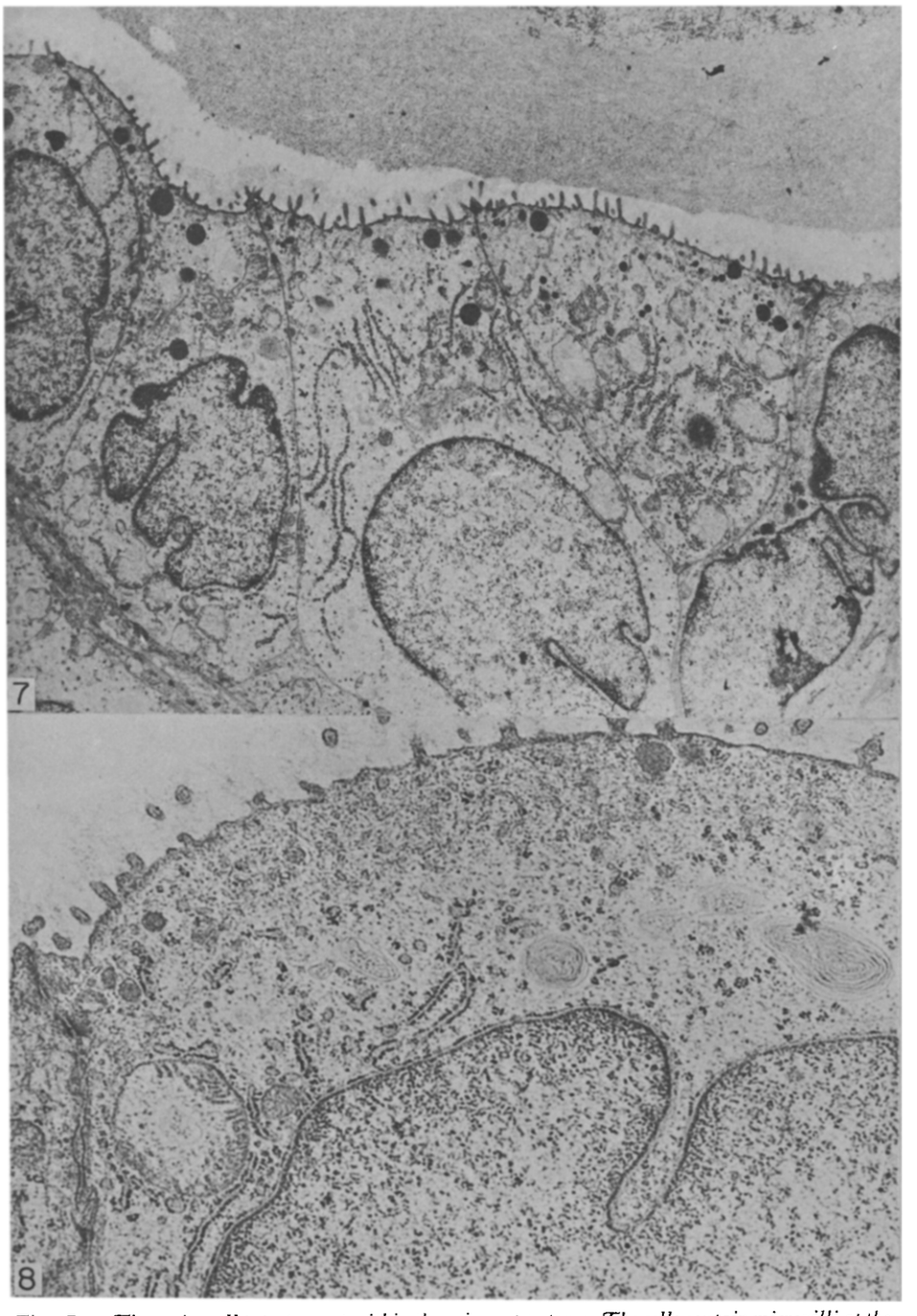

Fig. 7. The major cell type present within the acinar structures. The cells contain microvilli at the luminal margin, and have irregular nuclei which are often bilobed and usually contain clumped chromatin around the edges. Note the well developed Golgi zones, the myelin figures and the swollen mitochondria with irregular cristae only partially traversing the mitochondrial surface. The dense membrane limited granules present in the apical cytoplasm are mucous granules. Note the desmosomes and terminal bars connecting the cells. × 9300.

Fig. 8. Higher power view of the major cell type seen within the acinar structures. Note the prominent myelin figures (center, far right), swollen mitochondria, mucous granules (top), apical agranular endoplasmic reticulum (ER), free ribosomes and microvilli. × 15900.

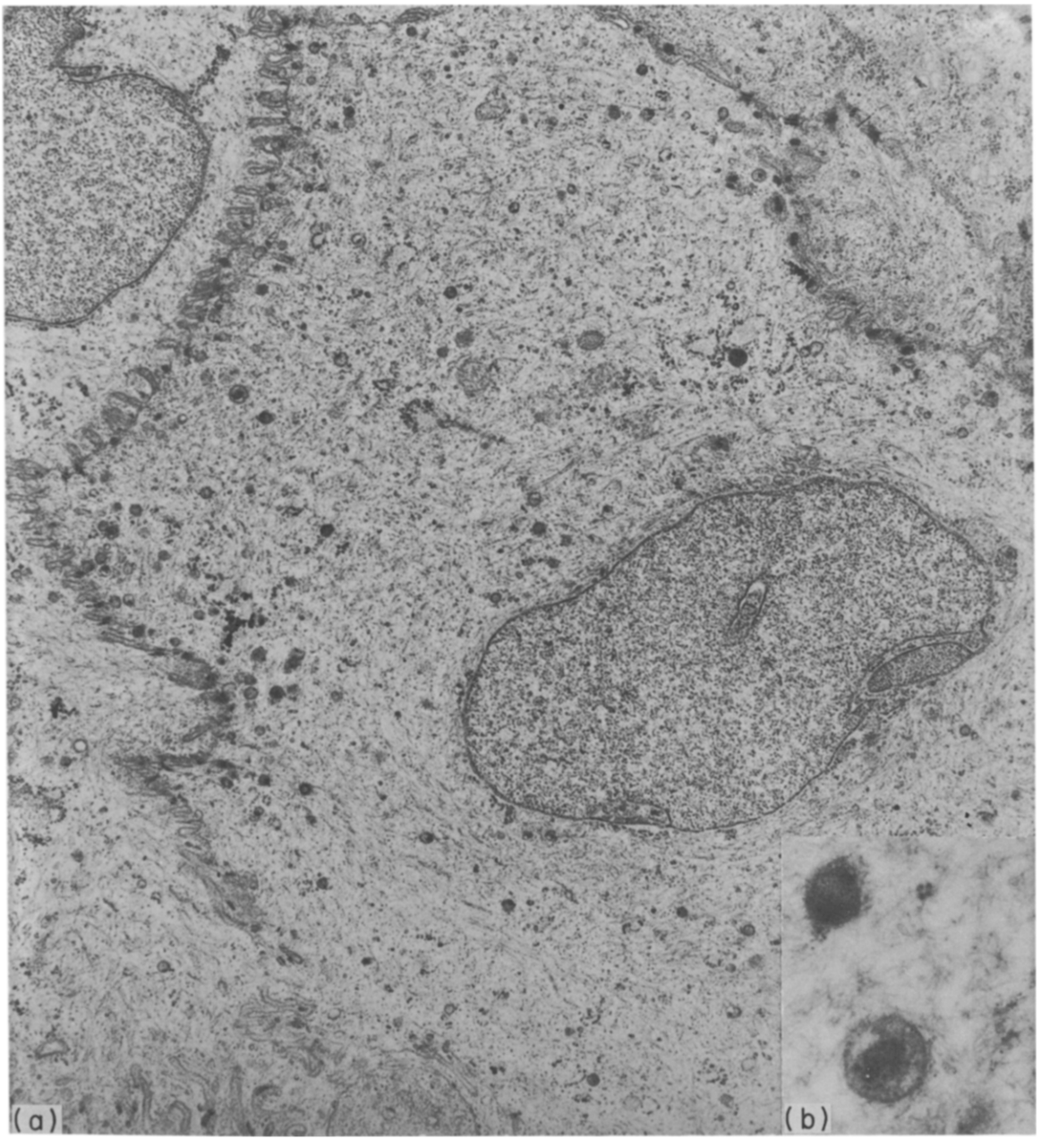

Fig. 9. (*a*) *Epidermoid type cells, showing complex interdigitations between cells, desmosomes, tonofibrils, many free ribosomes, and vesiculation of ER. These cells have very irregular nuclei with deep cytoplasmic invaginations. In many of the epidermoid cells, small round intracytoplasmic inclusion bodies are found* (*on the left side of the cell in center*). *The particles were all smaller than* 0.25 μm, *and are thought to be membrane coating granules* (*see text*). × 16,000. (*b*) (*Insert lower right*). *Higher power view of two membrane coating granules, as seen in Fig.* 9a. *Note that these particles have variable compositions, but often are seen with dense cores* (*lower left*). *The membrane coating granule appearing at the bottom of photomicrograph is the largest one found, slightly less than* 0.25 μm. × 40,415.

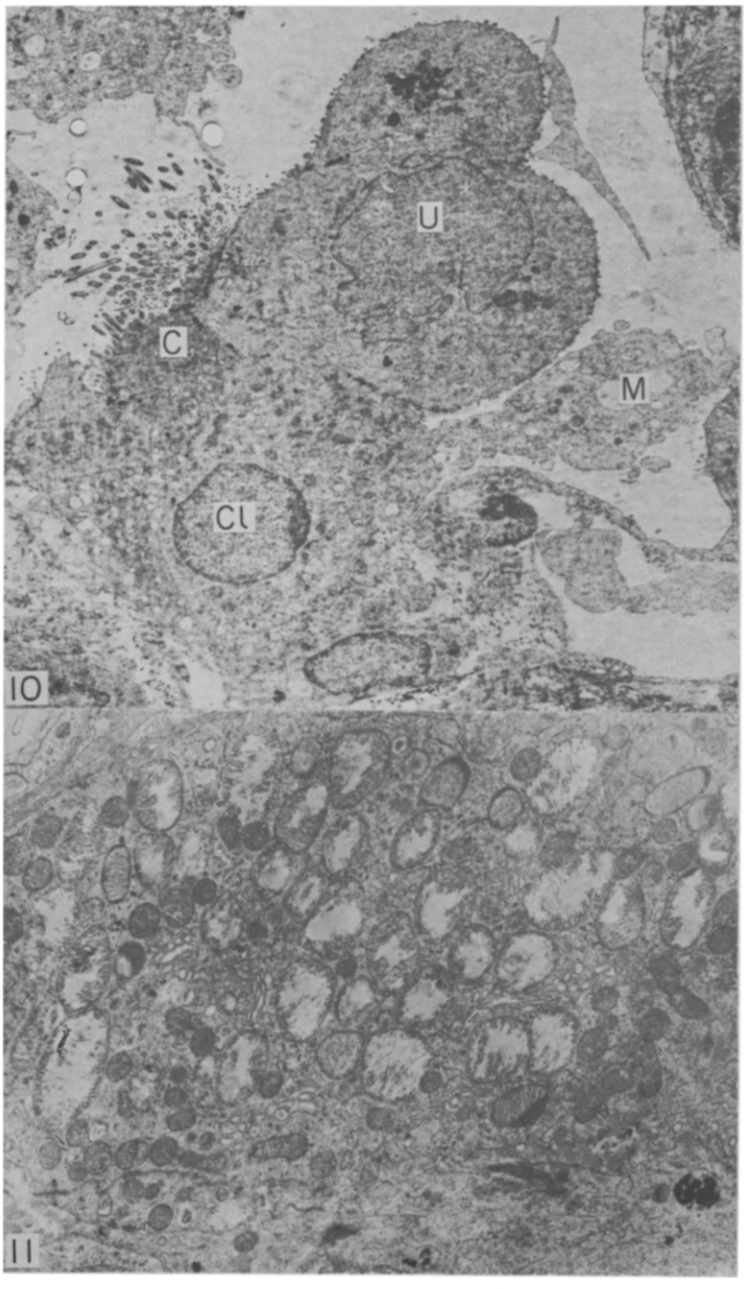

Fig. 10. *Four common cell types seen with the* ^{210}Po *induced tumors. At the top is an undifferentiated cell type (U) containing primarily ground substance, with microvilli, some granular ER and particulate glycogen, and always having an irregular nucleus with deep cytoplasmic invaginations. Below this cell and to the left is a ciliated cell (C), with typical cilia extending into the lumen. At the bottom-left is a cell resembling the Clara cell (Cl), with many granules containing electron lucent material, prominent microvilli, extensive supranuclear agranular ER, and a nucleus with condensed chromatin around the edges. A typical lung macrophage (M) can be seen at the right.* × 4133.

Fig. 11. *An enlarged type II alveolar epithelial cell containing many typical cytosomes, many small round mitochondria, several Golgi regions, and some free ribosomes. Such type II alveolar cells are seen scattered throughout the tumor region, and particularly around the edges of tumors.* × 6200.

ation of alveoli", a condition in which bronchiolar type cells line the preexisting alveolar walls. Ciliated and Clara cells, morphologically identical at the ultrastructural level to bronchiolar cells, suddenly appear in the alveolar region where they do not exist normally. This phenomenon has been observed after many unrelated injuries to the lung [30], including exposure to viruses [31, 32] and carcinogens [33]. It is not known whether these cells represent an abnormal extension of the bronchiolar epithelium or metaplasia of autochthonous alveolar epithelium, but the mechanism is thought to involve the colonization of alveoli with bronchiolar cells via bronchiolar–alveolar pores [30].

At a later stage in the epithelialization of alveoli, ciliated cells are not as prominent as cells resembling Clara cells. These Clara-like cells form acinar structures in the lung, or what we have called "hyperplastic nodules" [26], often with typical lung macrophages trapped inside. We consider these hyperplastic nodules a definite precursor lesion in tumor formation. It is interesting that Clara cell changes have been noted in other studies. A nodular hyperplasia of Clara-like cells at the bronchiolar–alveolar junction occurs after exposure to ozone in mice, and this is considered an irreversible lesion [34]. Hyperplasia of bronchiolar cells, presumably Clara cells, has also been observed after exposure to artificial smog [35] and virus infections [31, 35]. In separate autoradiographic studies accompanying our plastic section serial sacrifice experiment, Clara cells were shown to take up a significant fraction of the administered ^{210}Po [36].

In our studies, many of the Clara cells in the bronchioles, as well as the Clara-like cells in the acinar structures in the alveoli, begin to produce a PAS positive mucous secretion. Clara cells in the normal lung do not secrete granules that are PAS positive. There are a few reports that Clara cells do secrete a PAS positive secretion [13, 37, 38], but as we have pointed out [28] the confusion exists due to occasional PAS positive lysosomes and residual bodies present in the apical region of normal Clara cells. The real secretion product of Clara cells, seen as light secretion granules at the electron microscopic level [28, 39] is not PAS positive [28, 40, 41].

No cells, including Clara cells, produce mucus in human bronchioles; however a condition known as "goblet cell metaplasia", in which mucus secreting cells suddenly appear in the bronchiolar epithelium, occurs during chronic bronchitis [42] and is associated with respiratory failure [43]. It can be induced by experimental insults such as exposure to sulfur dioxide [42] and influenza virus infection [31, 35]. It is of interest that goblet cell metaplasia in bronchioles has also been observed in human lungs containing bronchiolar–alveolar carcinoma [13]. The term goblet cell metaplasia comes from paraffin section, light microscopic analysis of the phenomenon; an electron microscopic study of the cell type producing the PAS positive secretion apparently has not been done. In our study, the mucous cells in the tumors resemble goblet cells only by the nature of the secretion produced—they are not ultrastructurally normal hamster goblet cells (for a comparison, see reference [28]). How they arise is not clear. In man, it has been proposed that neurosecretory cells give rise to the goblet cell metaplasia [44]; however, neurosecretory cells do not exist in the hamster lung [28]. In our system, it is thought that these cells arise from preexisting Clara cells. Cycles of secretory products have also been observed in rat [42] and human [45] lung cells.

As is evident from the histochemical results, several mucous products are formed by the ^{210}Po induced tumors. Occasional cells produce sulfomucins or glycoproteins, while most of the secretory cells produce sialomucins or neutral mucopolysaccharides. All of these products are mucous secretions. Mucus is a general term used for a mixture of proteins and sugars, the chemical composition depending on the type of cell producing the mucus.

The acid product of the ^{210}Po induced tumor cells was shown to be a sialomucin which is resistant to neuraminidase (sialidase). Such a neuraminidase resistant sialomucin exists in the normal lung cells of the rat [46] and of man [45], and is also probably the main product of mucinous tumors of the trachea and bronchi in human lungs [47]. In our study, the neuraminidase resistant sialomucin was also found in a few atypical Clara cells in bronchioles near tumor regions, but most of the atypical Clara cells produced neutral mucopolysaccharides.

The cells in these ^{210}Po induced tumors apparently go through a cycle of secretion products. Using the AB pH 2.5–PAS staining sequence, the small lesions of the lung containing secretory acinar structures and squamous metaplastic lesions not connected with fully developed tumors contained only red material. Atypical Clara cells near tumor regions contained primarily red granules, although a few atypical Clara cells contained blue staining granules. Both red and blue granules appear in the tumor regions and only blue staining granules are found in the malignant cheek pouch tumors resulting from transplantation of the

^{210}Po induced tumors [48]. The red staining material is thought to be the simplest mucous secretion (neutral mucopolysaccharide), whereas blue granules are more complex products [45].

The changes in secretion products seen here are most likely occurring within a single cell type, as has been seen in other systems [42, 45]. After exposure to sulfur dioxide, rat lungs develop goblet cell metaplasia in the bronchioles and produce mucus which is at first sensitive to neuraminidase, but later becomes resistant to the enzyme [42]. It is thought that, in this case, a sialomucin appears first and later develops into a sulfomucin within the same cell. This change in the nature of the mucus also occurs after exposure to tobacco smoke and other irritants [42].

At later stages in tumor formation, some of the acinar structures containing the mucus-secreting cells became multi-layered and underwent squamous metaplasia. Squamous metaplasia in the lung is thought to be a reversible lesion which can be observed after chronic irritation [49, 50]. It has also been observed in experimentally produced lung cancers from ^{106}Ru [49] and polycyclic hydrocarbons [51]. In another study on peripheral lung tumors induced by pathogenic viruses, squamous metaplasia and the appearance of mucus-secreting cells in the alveoli were also considered early changes in the pathogenesis of squamous cell cancer [31].

At the electron microscopic level, the cells in the ^{210}Po induced tumors have many of the characteristics of glandular cells. In a well differentiated adenocarcinoma, the polarity of the individual cells is preserved, and desmosomes and terminal bars connect the cells as in normal bronchiolar epithelium [52]. This was commonly seen in the areas containing mucus-secreting cells. Other characteristics of adenocarcinoma cells include a well developed Golgi region [53], much rough endoplasmic reticulum [10, 54], microvilli [55], dark and light cells [54, 55], many mitochondria [52] which are often swollen [56, 57], mucus production [9], and "concentric membranes" [56]—here referred to as myelin figures. These are all common characteristics of the most prominent cell type present in ^{210}P0 induced tumors. In adenocarcinoma cells, the nuclear to cytoplasmic ratio is often small compared to other lung cancer cells [54]. This appeared to be the case for our ^{210}Po induced tumor cells, although the ratio was certainly large compared to normal bronchiolar or alveolar cells.

The ^{210}Po induced tumor cells have common characteristics of malignant cells in general, such as loss of the organized lamellar ergastoplasm, which is considered part of the dedifferentiation process occurring in cancer cells [57]. The ergastoplasmic membrane system is frequently altered in malignant cells so that the endoplasmic reticulum forms tubules or lamellae, and free ribosomes occur in the cytoplasm [57]. Both these phenomenon were seen in the mucus-secreting cells. These malignant cells are also transplantable, producing adenocarcinomas upon reinjection into hamsters [27, 48].

Mucus-secreting cells are not the only cell type present in ^{210}Po induced tumors. As seen by electron microscopy, there is a prominent epidermoid component as well. These cells have many desmosomes and tonofibrils, and a lack of cytoplasmic organelles such as mitochondria, endoplasmic reticulum and a Golgi complex. These are common characteristics of epidermoid differentiation seen in squamous cell carcinoma [52, 53, 56] and peripheral lung adenocarcinomas [10]. These epidermoid cells commonly had vesiculation of the endoplasmic reticulum, many free ribosomes in the cytoplasm, and cytoplasmic inclusion bodies with diameters less than 0.25 μm. These particles are probably "membrane-coating granules," which are known to develop within differentiating epithelial cells, move toward the cell periphery, fuse with the plasma membrane and empty their contents into the intercellular spaces [58]. These granules are considered specific differentiation products of epithelial cells.

Another cell type present in the tumors resembles Clara cells normally present in the bronchioles. There was extensive supranuclear agranular endoplasmic reticulum, many light secretion vesicles, and often bilobed nuclei with condensed chromatin around the edges; all of which are characteristics of Clara cells. Still another cell type, primarily composed of ground substance, appeared to contain glycogen particles, often a characteristic of normal Clara cells [29]. These undifferentiated cells had pleomorphic nuclei with deep cytoplasmic invaginations, a common characteristic of malignant cells [57]. These rather empty, anaplastic cells are typical examples of the dedifferentiation commonly occurring in neoplastic development [57].

Some of the cell types in the tumors were unique, with extraordinarily developed endoplasmic reticulum or Golgi apparatus, or with very long microvilli. These findings have been seen in lung cancer cells before and are thought to represent an incomplete maturation or differentiation of the cancer cells [54].

Normal ciliated cells and enlarged type II alveolar epithelial cells were also seen in these

tumors, but are thought to have little to do with the changes leading to malignancy. Ciliated cells have also been seen in other adenocarcinomas of the peripheral lung [7], but they are a rare occurrence in malignant tissue [57]. Nevertheless, some metastasizing tumor cells of hamster kidney origin have been known to form basal corpuscles of a fragmentary ciliated border [59]. When cilia are seen in bronchiolo–alveolar lung tumors, it is considered strong evidence for the cells of origin to be from bronchiolar epithelium [13].

The cells seen in the ^{210}Po induced tumors are very similar to the cell types seen at the electron microscopic level in the human peripheral lung carcinomas. For example, Mollo *et al.* [10] show a mucus-secreting cell (their Fig. 10) which looks ultrastructurally like our most prominent cell type (Figs. 7 and 8); their Fig. 9, a cell with light secretion granules resembling Clara cell granules, is very much like a cell type seen in our tumors (Fig. 10). Their tumors contained epidermoid cells with numerous desmosomes and tonofilaments (their Fig. 11) as seen in our Fig. 9. Kuhn [13] and Greenberg *et al.* [14] noted cells with glycogen granules similar to one of our cell types [Fig. 10] and Bedrossian *et al.* [60] and Geller and Toker [55] also described dark and light cells. These cells, commonly found in human bronchiolar–alveolar carcinomas, are considered indistinguishable at the electron microscopic level from the cells in well differentiated adenocarcinomas arising more centrally in the bronchial tree [60].

A wide variety of cell types might be expected in these tumors. When cheek pouch tumors are grown from cloned malignant cell lines derived from these ^{210}Po induced peripheral lung tumors [27, 48] the resultant tumors are made up of several different cell types. Thus, many of the cell types seen in the tumors discussed here could have come from a single transformed cell. The multipotential nature of the stem cells of squamous cell carcinoma, in which a single cell type can give rise to many types of tissue, has been shown previously by Pierce and Wallace [61].

The variety of cell types present in these tumors may help explain the different results obtained in the histochemical studies done here. The tumor areas present in the lungs studied showed a variety of secretion products, with the most frequent product being of a mucoid nature, as has been discussed. The frozen section studies showed some areas of the tumors, and some regions around the edges of tumors, to have sudanophilic areas shown to be phospholipid by Baker's acid hematein method. In the alveolar region of the normal lung, type II alveolar epithelial cells produce a substance of phospholipid nature [22, 62]; however, there is also evidence that Clara cells of the bronchioles may produce such a secretion [38, 40, 41, 63]. In our studies, Clara cells in the bronchioles were not normally Sudan black positive; however, in bronchioles near tumor regions, some Clara cells did produce sudanophilic granules. Some Sudan black positive results have been reported for normal Clara cells [38, 40]. Our lack of a sudanophilic reaction could be due to species' variations in Clara cells (for discussion, see reference [28]). Thus, normal type II alveolar epithelial cells, the hyperplastic and hypertrophic type II alveolar epithelial cells, or the cells resembling Clara cells with many secretion granules could be producing the phospholipid secretion found in our tumors.

The production of surfactant has been used by other workers to determine the cell of origin of bronchiolar–alveolar carcinomas. It has not been found in two studies of bronchiolar–alveolar carcinoma [13, 64], or in secretions obtained from a bronchiolar–alveolar carcinoma by bronchial lavage [65]. These studies related the histogenesis of the tumors to bronchiolar epithelium.

We have observed that the Clara cells in bronchioles of tumor-containing lungs undergo a variety of changes after ^{210}Po exposure, such as becoming sudanophilic or PAS positive in discrete areas. Some of the ultimate tumor cells are sudanophilic, some are PAS positive. In a separate study of enzyme reactions of normal and tumor-containing lungs, it was noted that the enzyme reactions occurring in the ultimate tumor cells most resembled those of normal Clara cells [66]. A significant finding was that Clara cells in bronchioles of tumor-containing lungs began producing large amounts of alkaline phosphatase, and alkaline phosphatase activity was high in the cells of the tumor region [66]. Clara cells originally present in the bronchioles and appearing in the alveolar region after ^{210}Po exposure may go through changes which ultimately lead to a malignant tumor. The normal secretion product of Clara cells is produced in great quantities in some of the bronchiolar Clara cells after ^{210}Po exposure, in Clara-like cells in hyperplastic nodules appearing in the alveoli, and in some of the ultimate tumor cells, as observed here ultrastructurally and histochemically. These cells are probably not malignant cells. However, bronchiolar Clara cells exposed to ^{210}Po, the cells in the hyperplastic nodules and the tumor cells also produce the same PAS positive secretions, neutral mucopolysaccharides

and neuraminidase-resistant sialomucins. Ultrastructurally, the mucus-secreting tumor cells still have a few basic characteristics of Clara cells [63]; e.g. apical agranular endoplasmic reticulum and often bilobed nuclei with condensed chromatin around the edges. Bronchiolar cells have been implicated previously as the cells of origin of bronchiolar–alveolar carcinoma [55]. Recent ultrastructural and strong histochemical evidence not discussed here have also singled out the Clara cells of the bronchioles as the cells of origin of human bronchiolar–alveolar carcinoma [13, 14]. That a tumor should arise in the alveolar region with bronchiolar characteristics is not surprising since the alveoli and bronchioles have a common embryologic origin [39, 67, 68]. It has been proposed that typical bronchiolar cells can colonize the alveoli through bronchiolo–alveolar pores [30], so the appearance of a carcinoma with bronchiolar cells in the alveolar region is conceivable. The most likely explanation is that the Clara cells of the respiratory bronchioles originate the tumors. The hamster respiratory bronchiole consists of very short stretches (only a few cells) of bronchiolar epithelium, primarily Clara cells, with extensive alveolar outpocketings [28], and thus appears histologically more like the alveolar region than the bronchiolar region.

Our conclusions about the histogenesis of these peripheral lung tumors are different from those reached by several other investigators studying such tumors. Adamson *et al.* [15], Nagaishi *et al.*[53] and Coalson *et al.*[12] have concluded that type II alveolar epithelial cells are the cells of origin of human bronchiolar–alveolar carcinoma, and several other studies such as those of Brooks [16], Klarner and Gieseking [17] and Okada *et al.*[69] have attributed the origin of peripheral lung tumors in animals to type II alveolar epithelial cells.

In our ^{210}Po serial sacrifice study, major early changes in type II alveolar epithelial cells were noted. These cells responded to the treatment by undergoing hyperplasia and hypertrophy with an increased number of cytoplasmic inclusions or cytosomes, resulting in foci that were composed entirely of type II alveolar epithelial cells. This response of the type II alveolar epithelial cells has been noted after a variety of injuries to the lung, including chronic irritation [70], nitrogen dioxide exposure [71], and drugs [72]. Enlarged type II alveolar epithelial cells continued to be conspicuous during and after the formation of the tumor, being seen in large numbers around the edges of the ^{210}Po induced tumors and scattered throughout the tumors. However, we do not feel that type II alveolar epithelial cells are involved in the ultimate formation of the tumor for three reasons: (1) The type II alveolar epithelial cells do not appear to be involved in the lesions of the lung we consider pre-neoplastic; (2) the tumor cells do not contain typical or atypical type II alveolar epithelial cell cytosomes; and (3) no cells resembling type II alveolar epithelial cells (i.e. containing cytosomes) grow out after the tumors have been transplanted [27, 48]. The prominent cell type in the ^{210}Po induced tumors contains myelin figures (Fig. 8) which might be mistaken for cytosomes (Fig. 11). Myelin figures, being composed of phospholipid [73] as are cytosomes [22, 62], are easily induced in Clara cells by exposure to ozone [34], chlorphentermine [74], and Iprindole [75]. We have seen small numbers of myelin figures in normal hamster Clara cells [28]. Sommers *et al.* [64] describe myelin figures in adenocarcinoma cells presumably derived from bronchiolar epithelium.

Three other recent studies [13, 14, 60], which attribute the origin of bronchiolar–alveolar carcinomas to bronchiolar epithelial cells, have also questioned previous conclusions that type II alveolar epithelial cells are the cells of origin. To explain the difficulty in determining the cell of origin, Greenberg *et al.* [14] point out that atypical areas of type II alveolar epithelial cells adjacent to tumors can often be confused with the tumor itself. In the studies of Coalson *et al.* [12], Nagaishi *et al.* [53] and Adamson *et al.* [15], which attribute the origin of bronchiolar–alveolar carcinomas to type II alveolar epithelial cells, it is impossible to tell the relationship of the atypical type II alveolar epithelial cells to the tumor since 1 μm sections are not shown. In some studies which attribute the origin of the tumors to type II alveolar epithelial cells, atypical cells resembling Clara cells and mucus-secreting cells can be seen in the tumor photomicrographs shown along with the atypical type II alveolar epithelial cells (see, for example, Okada *et al.* [69], Figs. 8 and 9; Nagaishi *et al.* [53], Figs. 2 and 11). Mollo *et al.* [10] attributed the development of some bronchiolar–alveolar carcinomas to type II alveolar epithelial cells; in other tumors, in which mucus production or an epidermoid component was prominent, the histogenesis was unclear. In systems other than the ^{210}Po induced tumors, type II alveolar epithelial cells may be the precursors for the malignant cells. They are considered the target cells for urethane-induced tumors in mice [16, 76], and the cells of origin of spontaneous lung tumors such as those in mice [16, 17] and sheep [18]. However, in a recent comprehensive review of all electron microscopic information

published about the origin of bronchiolar–alveolar carcinomas. Bedrossian *et al.* [60] concluded that if a neoplasm of great alveolar cells exists, definite documentation of its occurrence is still lacking.

Our studies strongly suggest that the Clara cell is the cell of origin of ^{210}Po induced peripheral lung tumors. These tumors have histopathological features observed in human bronchiolar–alveolar carcinomas [9–14] and in many species of animals after exposure to chemical carcinogens [77], alpha-emitting radioisotopes [19] and beta–gamma emitters [78], and oncogenic viruses [31]. ^{210}Po radiation doses to Clara cells can be calculated to obtain an estimate of risk for the induction of malignant transformation in this sensitive cell type. Similar calculations for the radiation doses from the plutonium isotopes might explain the differences in tumor incidence with varying methods of administration. Plutonium isotopes, which emit alpha particles similar in energy and path length to ^{210}Po, produce primarily bronchiolar–alveolar carcinomas in a wide variety of animal species and after many types of exposure conditions [19]. When injected intravascularly on zirconium oxide microspheres, however, plutonium produces minimal pathologic changes and few lung tumors [79]. The microspheres lodge in the peripheral lung capillaries of the alveolar septa and remain there as fixed radiation sources. Since the alpha particle track length in tissue is very short (approximately 40 μm), most of the radiation dose following this method of exposure may not reach the sensitive Clara cells of the terminal and respiratory bronchioles. Similarly, hamsters exposed to plutonium isotope aerosols [80] failed to develop lung cancer (although some of the hamsters in this study did have lesions we consider premalignant), perhaps because only a small fraction of the alpha radiation dose from inhaled plutonium isotopes actually reaches the terminal bronchiolar cells [81]. Indeed, the lack of tumors in both of these studies, as compared with the known lung tumor-inducing potential of plutonium [19], may be explained by differences in the distribution of radiation dose to the critical cell population. Thus, knowledge of the target cell for transformation may be particularly important for estimating the carcinogenic hazard from inhaled plutonium isotopes produced by nuclear power plants as well as from many chemical agents.

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Morphologic and Histochemical Characteristics of Cell Lines Derived from Hamster Peripheral Lung Tumors*

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Abstract—*Intratracheal instillation of the alpha emitter polonium-*210 (*^{210}Po*) *or benzo[a]pyrene induces peripheral lung tumors in Syrian hamsters which have a combined epidermoid and adenomatous morphology similar in appearance to human bronchiolar–alveolar carcinoma. Two permanent cell lines derived from such tumors have been studied in an attempt to determine the cell of origin of the original peripheral tumors. The ultrastructural and histochemical characteristics of both the cells and of cheek pouch tumors grown from HLAC-*4 *and HLAC-*14 *cells at various stages of passage in tissue culture in general support, but do not prove, our hypothesis that Clara cells found in the terminal and respiratory bronchioles are the cells of origin of the peripheral lung tumors induced in hamsters by these carcinogens.*

INTRODUCTION

As PART of a series of experiments designed to determine the cell of origin of ^{210}Po induced tumors in the peripheral hamster lung [1], we have studied the morphology of the cell lines originally derived from these tumors. The isolation and passaging of the malignant cell lines, both *in vivo* and *in vitro*, have been discussed by Terzaghi and Little [2]. Terzaghi and Little [2] also presented a brief description of the light microscopic morphology of the cell lines HLAC-4 and HLAC-14, the cell lines used for this more extensive morphologic study. The HLAC-4 and HLAC-14 cell lines were originally derived from combined epidermoid and adenocarcinomas of the peripheral lung induced by ^{210}Po or ^{210}Po and benzo[a]pyrene (BP). An examination of paraffin sections of tumors resulting from the inoculation of cells from either of these lines into hamster cheek pouches revealed that the epidermoid differentiation present in the primary tumor [3] did not occur in the cheek pouch tumors. Thus, there was an exclusive emergence of adenomatous and undifferentiated cell types in the cheek pouch tumors [2]. In the present investigation, we have examined the ultrastructural and histochemical characteristics of the cells in these cell lines in order to identify the cell which initiates the hamster lung peripheral tumors.

MATERIAL AND METHODS

The isolation and passaging of neoplastic cell lines, originally derived from peripheral hamster lung tumors induced by ^{210}Po or ^{210}Po and BP, have been described in detail by Terzaghi and Little [2]. Two of these tumor cell lines. designated HLAC-4 and HLAC-14, were studied here by both light and electron microscopy. Cellular morphology was studied using plastic sections for light and electron microscopy as described below and with standard paraffin embedded sections of alcoholic-zinc formalin fixed, cheek pouch tumors produced by cells at different stages of *in vivo* and *in vitro* passage. Determination of the nature of the mucin in the acinar structures of cheek pouch tumors at early

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passages of the cell lines was done on paraffin sections using the Alcian blue (AB) pH 2.5–periodic acid–Schiff (PAS) sequence [4], the AB pH 1.0–PAS sequence [4], and the high iron method [5].

For electron microscope examination, malignant cheek pouch tumors were grown from cells treated in each of the following ways: (1) HLAC-4 cells, which had been sequentially cloned [2] were grown *in vitro* for a total of 7 months. The cells originally isolated from a primary lung tumor were initially passed through a hamster cheek pouch, then passed in tissue culture for 5 months, passed through a hamster cheek pouch, and returned to tissue culture for an additional 2 months when used for electron microscopy. (2) HLAC-14 cells, like the HLAC-4 cells, were originally passed from the primary lung tumor into a hamster cheek pouch. A section of this original cheek pouch tumor was used for electron microscopic examination. Cells which had been sequentially cloned *in vitro* were then inoculated into a cheek pouch after 1 month in tissue culture, and after the HLAC-14 cells had been in tissue culture for 6 months.

HLAC-4 cells growing *in vitro* were also examined electron microscopically. After the initial passage through a hamster cheek pouch, exponentially growing cells were kept in tissue culture for 2 months before being taken for electron microscope examination.

Preparation of cheek pouch tumors for electron microscopy was as follows. Sections of tumors less than 1 mm^2 were placed in Karnovsky's glutaraldehyde–paraformaldehyde fixative [6] for 3 hr, then washed 4 times in cacodylate buffer (0.1 M, pH 7.4), and stored overnight in cold buffer. The blocks were then post-fixed in s-collidine-buffered osmium tetroxide (1.3% osmium tetroxide, 0.1 M s-collidine, pH 7.4) for 1 hr, washed 4 times in s-collidine buffer, and dehydrated and embedded in epon. For electron microscopic examination of the HLAC-4 cells *in vitro*, the preparation techniques of Gimbrone, Cotran and Folkman [7] were used. Briefly, cells growing exponentially in 60 × 15 mm Falcon tissue culture dishes were fixed in 25% glutaraldehyde in 0.1 M sodiumcacodylate buffer with $CaCl_2$, post-fixed in 2% osmium tetroxide and embedded in epon. Tissue sections were cut on a Reichert ultramicrotome. One micron sections were stained with toluidine blue. Appropriate blocks were trimmed for electron microscopy, silver sections cut with a diamond knife (DuPont), mounted on 300 mesh copper grids, and stained with uranyl acetate and lead citrate. Sections were examined with a Philips 300 electron microscope.

RESULTS

Light microscopy

When injected into hamster cheek pouches, both cell lines produced cheek pouch tumors having obvious adenomatous and undifferentiated components at the light microscope level (Figs. 1–3).* This included acinar structures resembling bronchiolar epithelium (Fig. 2) which appeared in varying amounts in cheek pouch tumors resulting from the injection of cells of either cell line at both early and late passages. When both original tumors were transplanted directly into cheek pouches and grown out, the cells retained the ability to produce a PAS positive secretion. This ability was lost, however, with continued passage through hamster cheek pouches. When either of the cell lines which had been continuously carried in tissue culture were injected into hamster cheek pouches, the cells in the acinar structures for the most part did not produce a PAS positive secretion.

To determine the nature of the PAS positive secretion in the early cheek pouch tumors, mucin stains were done with the following results:

AB pH 2.5–PAS sequence. This staining sequence distinguishes neutral (red) from acidic (blue) mucopolysaccharides. The acinar structures contained blue material within and blue granules in the epithelial cells. Blue nuclear staining is due to nucleic acids. Undifferentiated cells were red.

AB pH 1.0–PAS sequence. Neutral mucosubstances are still red, but alcinophilic substances at this pH include only sulfated mucopolysaccharides. Material within the acinar structures was red, and red granules were present in the cells within the structures. Some of the acinar structures showed cells with a very thin red and blue rim.

High iron method—to distinguish sulfomucins. Mostly negative results, with only a few positive cells in the acinar structures.

Electron microscopic examination

The cheek pouch tumors which grew out after the injection of cells from either of the cell lines were quite similar; and with the HLAC-14 cell line, the ultrastructural morphology did not markedly change from the original cheek pouch tumor to the tumors produced by cells that had been in tissue culture for 1–6 months.

*Figures 1–3 are light microscopic views of a cheek pouch tumor grown from a piece of a primary tumor in a hamster lung. The established cell line from the original tumor was designated HLAC-14.

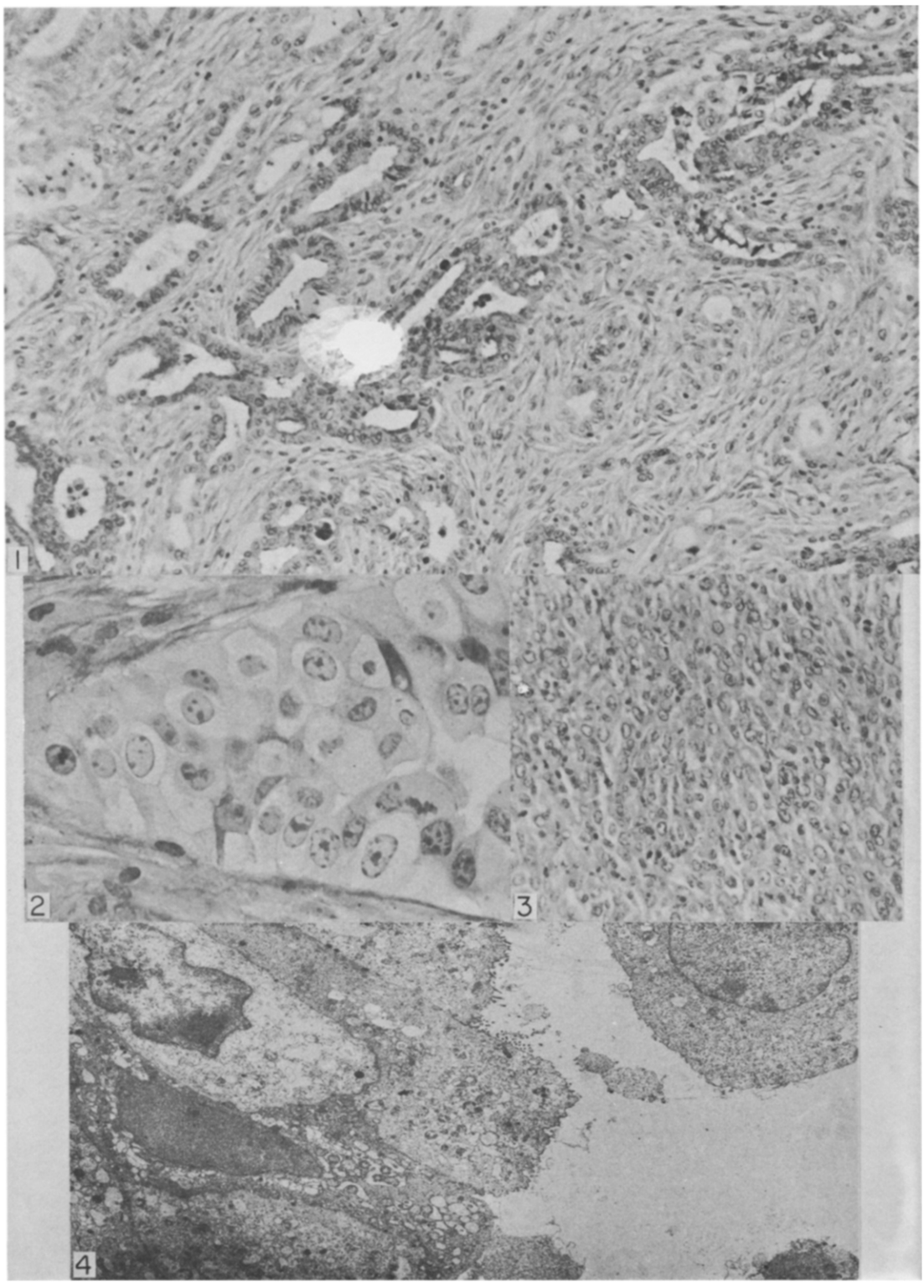

Fig. 1. *Low power view of paraffin embedded tissue: adenomatous (center) and undifferentiated (bottom right) cell types occur in these cheek pouch tumors. A PAS positive mucous secretion is produced by some of the cells in the adenomatous areas (center). PAS-hematoxylin.* × 200.

Fig. 2. *High power view of cell within adenomatous area. Note that both dark and light cells occur frequently in the glandular structures. Nuclei contain prominent nucleoli. One micron epon section, stained with toluidine blue.* × 500.

Fig. 3. *Paraffin embedded section of cheek pouch tumor showing undifferentiated sheets of cells. PAS-hematoxylin.* × 250.

Fig. 4. *HLAC-14 cell line. Cells within an acinar structure in a cheek pouch tumor produced by cloned HLAC-14 cells in tissue culture for one month. Note that both dark and light cells exist in these structures. The dark cells are large, with irregular microvilli. They usually contain large, homogeneous and electron dense nuclei (center left). There is extensive development of smooth and rough endoplasmic reticulum as well as many free ribosomes. The light cell type (top left) has very few cytoplasmic organelles compared to the dark cell type. There is some rough endoplasmic reticulum often arranged into vesicles (apical region of cell). Free ribosomes are also present, and occasional lysosomes (apical region of cell) can be seen. There are two nucleoli, and the nucleus contains both electron dense and electron lucent areas with a thin peripheral clumping of chromatin.* × 3616.

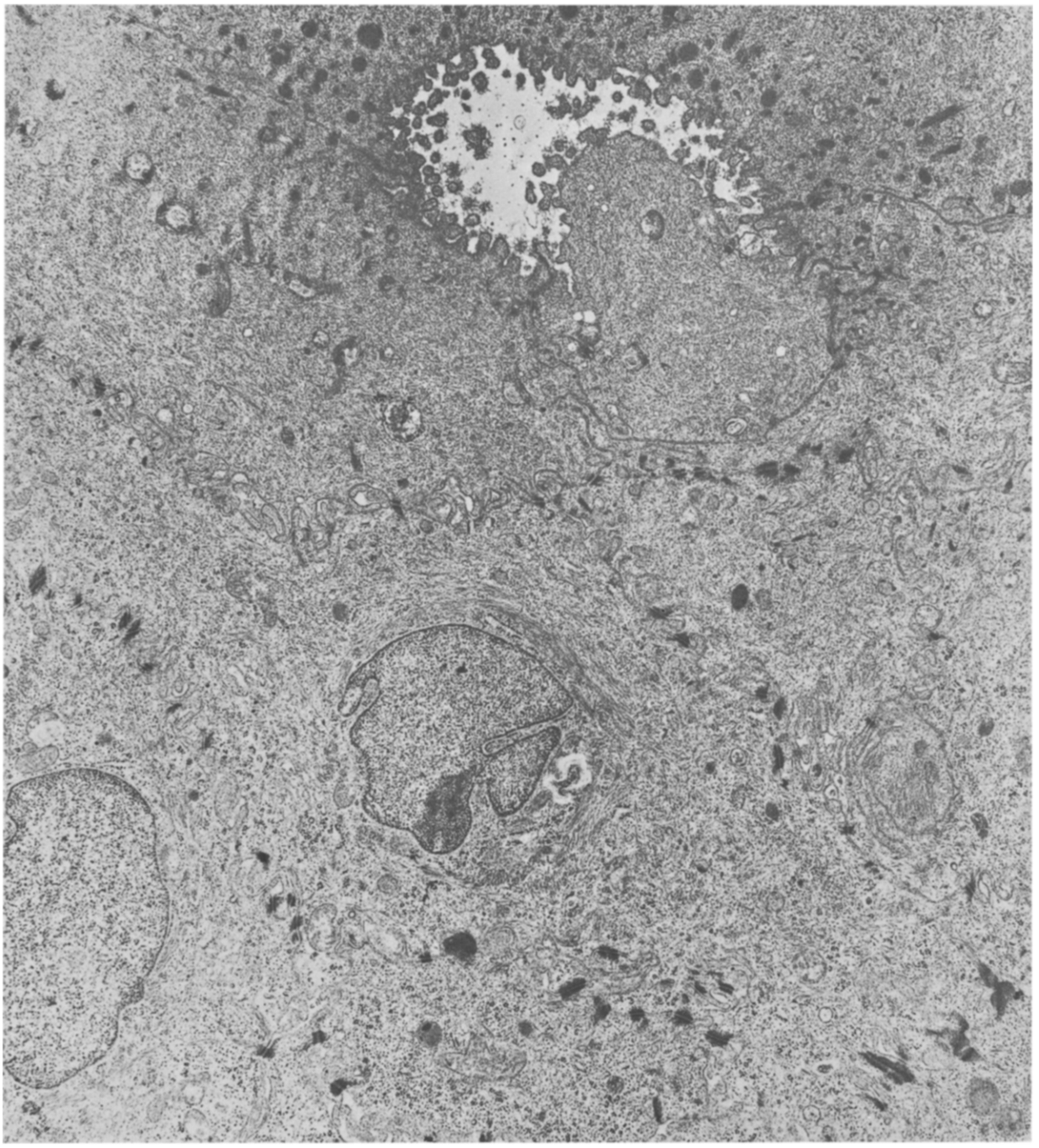

Fig. 5. Epidermoid cells growing within a cheek pouch tumor grown from the HLAC-14 cell line after 6 months in tissue culture. The epidermoid cells contain numerous tonofilaments (center) and free ribosomes, and irregular, uniformly dense nuclei with deep cytoplasmic invaginations and prominent nucleoli. Cell to cell connections include numerous desmosomes and complex interdigitations between cells. × 12400.

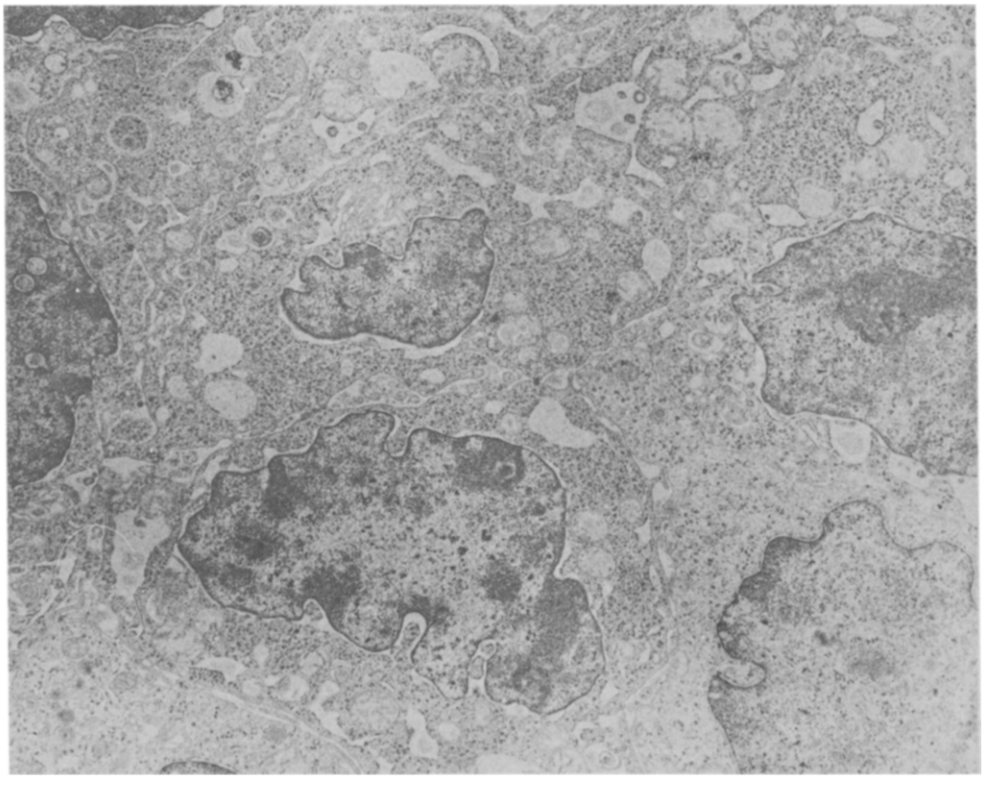

Fig. 6. *HLAC-4 cell line. Undifferentiated cells seen in a cheek pouch tumor produced by cells which had been in tissue culture for 7 months. Note the extensive dilation and vesiculation of rough endoplasmic reticulum, many free ribosomes, and many swollen mitochondria with cristae only partially traversing the mitochondria. Note the well developed Golgi region (near top, center). There is a large nuclear to cytoplasmic ratio, and the nuclei have large cytoplasmic invaginations and prominent nucleoli. An occasional microvillus can be seen, and there are occasional desmosomes (center) between cells.* × 9300.

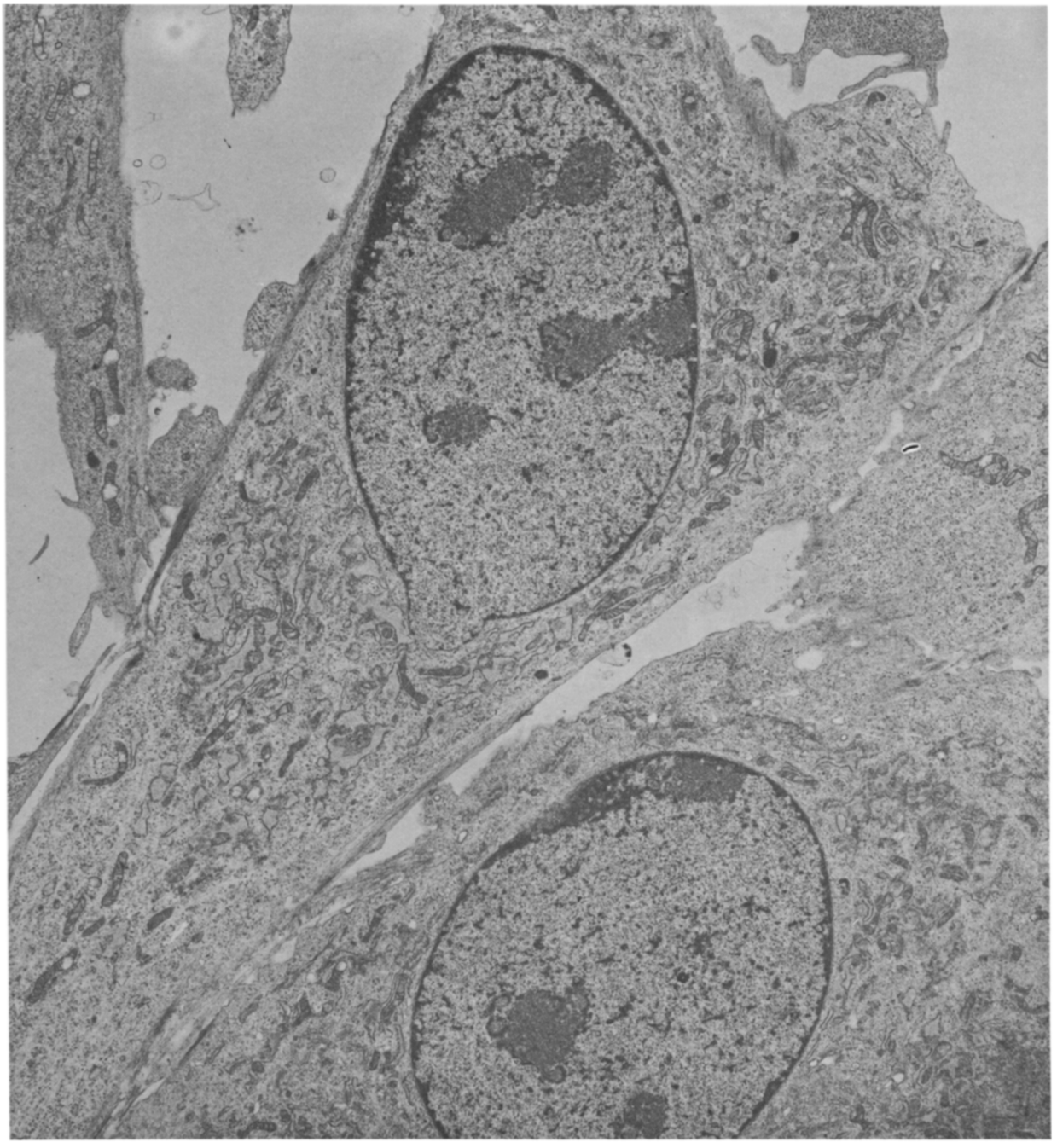

Fig. 7. HLAC-4 cells growing in vitro. *Note the regular, oval nuclei containing multiple, prominent nucleoli. There is extensive development of endoplasmic reticulum, both granular and agranular. Vesicles of granular endoplasmic reticulum are common, and there are many free ribosomes. Oblong mitochondria are numerous.* × 6000.

1. *HLAC-14 cell line* (*Figs.* 4–5). Results from the original cheek pouch tumor, and tumors produced by cells that had been in tissue culture for 1–6 months.

The cells in the acinar structures all had microvilli, and were large with pleomorphic nuclei having deep cytoplasmic invaginations and prominent nucleoli. Cell to cell connections included desmosomes and terminal bars. There were two basic cell types in the acinar structures—one was "dark" and had extensive development of smooth and rough endoplasmic reticulum, and many free ribosomes (Fig. 4). There were numerous round and oblong mitochondria, rich in matrix and often swollen with irregular cristae which only partially traversed the mitochondria. There were also many degenerating mitochondria. There were lysosomes in the basal to mid-portion of the cells, and occasional myelin figures in the apical region. There was a complete loss of organized lamellar ergastoplasm. In many cells, there was dilation and extensive vesiculation of the rough endoplasmic reticulum into round or irregular structures containing a material of moderate electron density, but in most cases the center of these structures was clear. In some cases, these structures contained electron dense cores or myelin figures. There was also much smooth endoplasmic reticulum and many free ribosomes contributing to the dark cytoplasmic matrix. In the original cheek pouch tumor, there were small round mucous secretion granules of moderate electron density—they were not seen in the other cheek pouch tumors studied at the electron microscopic level.

The other cell type in the acinar structures was "light" (Fig. 4) and had very few cytoplasmic organelles compared to the darker cells. These cells had very little rough endoplasmic reticulum, but when present was often arranged into irregular structures. There were occasional swollen mitochondria with irregular cristae which only partially traversed the mitochondria. There were occasional free ribosomes and some lysosomes. There were often multiple nucleoli in which the nucleolonema was prominent, and the nuclei always had electron lucent and electron dense areas with a thin peripheral clumping of chromatin. These same basic cell types were seen in the acinar structures at all three times studied, and their morphology remained relatively constant aside from the loss of secretion granules after prolonged passage of the original cheek pouch tumor.

Cell types found in the cheek pouch tumours other than in the acinar structures included epidermoid cells with many tonofibrils and desmosomes and cell borders containing complex interdigitations between cells (Fig. 5). These cells were very electron dense with many free ribosomes and some mitochondria, primarily round and rich in matrix. The nuclei were uniformly dense with multiple nucleoli and deep cytoplasmic invaginations. Interspersed among these epidermoid cells were "light" cells, which are very much like the light cells seen in the acinar structures described above. Desmosomes were not seen in the cell to cell borders of light cells.

Undifferentiated cell types contained much rough endoplasmic reticulum, many free ribosomes, and numerous swollen mitochondria with irregular cristae. Vesicles of endoplasmic reticulum often contained myelin figures. There was often a well-developed Golgi region. There were occasional microvilli and desmosomes between cells. Nuclear membranes were deeply infolded, and nuclei contained multiple nucleoli with prominent nucleolonemae. Cells similar to these but having a "lighter" cytoplasm, due primarily to the lack of many free ribosomes and agranular endoplasmic reticulum in the cytoplasmic matrix, were also seen.

2. *HLAC-4 cell line.* Within the acinar structures of cheek pouch tumors formed by the HLAC-4 line cells, the cells were quite uniform, and were quite similar at the electron microscopic level to the "dark" cell described above for the HLAC-14 cell line acinar structures.

Undifferentiated cells are shown in Fig. 6. There was extensive dilation and vesiculation of rough endoplasmic reticulum, often with myelin figures appearing within the vesicles, many free ribosomes, often a well developed Golgi region, and many swollen mitochondria with irregular cristae partially traversing the mitochondria. There was a very large nuclear to cytoplasmic ratio, and the nuclei had deep cytoplasmic invaginations. Occasional microvilli were seen. There were occasional desmosomes between these cells, and intercellular spaces were often small.

These two major cell types seen in the HLAC-4 cell line were basically similar to two of the cell types seen in the HLAC-14 cell line. However, the HLAC-4 cell line lacked both an epidermoid component and those cells referred to as light cells in the HLAC-14 cell line.

HLAC-4 cells growing *in vitro* are shown in Fig. 7. The cells were quite homogeneous in culture, containing electron dense, oval nuclei with multiple prominent nucleoli. There were many free ribosomes and much endoplasmic reticulum, both granular and agranular. Vesicles of endoplasmic reticulum could also be

found. Oblong mitochondria were numerous, often with cristae only partially traversing the mitochondria. There were occasional myelin figures. There were some desmosomes between cells and a few microvilli could be found.

DISCUSSION

The cells seen in these cheek pouch tumors have many of the characteristics often associated with malignant cells, including large pleomorphic nuclei with a deeply infolded nuclear membrane [8], prominent nucleoli as large as 3 μm with a distinct nucleolonema [9], and loss of the organized lamellar ergastoplasm with an increased number of free ribosomes throughout the cytoplasm [8]. In malignant cells, the ergastoplasmic membrane system is frequently altered so that tubules or lamellae form vesicles which often contain a homogeneous substance of medium electron density [8], as was commonly seen in the cheek pouch tumor cells. Bernhard [8] considers the vesiculation of endoplasmic reticulum an example of the dedifferentiation process occurring in malignant cells. In addition, most of the cells in the HLAC-4 and HLAC-14 cheek pouch tumors have malignant characteristics associated with adenocarcinomas. In a well differentiated adenocarcinoma, the polarity of individual cells is well preserved, as in normal bronchiolar epithelium, with desmosomes and terminal bars connecting the cells [10]. This is seen in the gland-like, acinar structures of both the HLAC-4 and HLAC-14 cell lines. Many cheek pouch tumor cells in areas without acinar structure were arranged irregularly and without distinct polarity, a characteristic of poorly differentiated adenocarcinoma. The cells in these areas have been termed undifferentiated at the light microscope level [2], but at the electron microscope level they actually have many of the ultrastructural characteristics common to adenocarcinoma cells. These include a well-developed Golgi region [9]; much rough endoplasmic reticulum which often forms enlarged cisternae and is frequently filled with granular material [11, 12]; microvilli [13]; dark and light cells [13, 14]; many mitochondria [10] which are often swollen [15]; and "concentric membranes" [15], here referred to as myelin figures. Mucus production, characteristic of adenocarcinomas *in vivo* [16], is also present in the gland-like structures of cheek pouch tumors at early cell passages. Although most of the cells in both cell lines have such adenomatous characteristics, some of the cells in the HLAC-14 cell line have characteristics associated with epidermoid carcinomas, including numerous desmosomes and tonofibrils and a lack of cytoplasmic organelles such as mitochondria, endoplasmic reticulum, or a Golgi complex [10, 15].

Although the cells in these tumor lines have many of the characteristics of malignant cells at the electron microscope level, proof of their malignancy can not be determined from electron micrographs. Unfortunately, specimens of tumors from which these cell lines were isolated were not obtained [2]; however, in subsequent studies, sections of transplanted tissue showed clear evidence of combined epidermoid and adenocarcinomas (unpublished data). The question always arises as to whether transplantation in the cheek pouch is evidence of malignancy. Three characteristics of these cell lines growing in cheek pouches are indicative of malignancy: (1) The cheek pouch tumors do not regress [2] as they would if they were derived from normal cells [17]; (2) the cheek pouch tumors resulting from growth of these cell lines can be passed to other hamsters [2, 17]; and (3) a cell inoculum of only 500 cells of these cell lines leads to cheek pouch tumors [2]; "normal" cells require a critical inoculum of $>10^4$ cells [17]. Furthermore, upon reinjection into hamsters via intraperitoneal or intratracheal routes, the HLAC cells lead to widespread metastatic and invasive tumors, and their *in vitro* growth characteristics are those of transformed cells [2].

The HLAC-4 cells growing *in vitro* also showed many of the malignant and adenomatous characteristics described above. However, these cells did not have the swollen mitochondria characteristic of the cheek pouch tumor cells grown from the injection of HLAC-4 cells. The cheek pouch tumors from which the electron microscopic sections were taken were quite large (about 0.5 cm), and many of the cells studied showed some evidence of degeneration. Swollen mitochondria are often seen in degenerating cells [18], so it seems likely that this characteristic, commonly attributed to adenocarcinoma cells [15] is really evidence of degeneration in our cheek pouch tumor cells. Dilation and vesiculation of endoplasmic reticulum are also considered evidence of degeneration [18]. This phenomenon was seen in both the HLAC-4 cells growing *in vitro* and in the cheek pouch tumor cells grown from the HLAC-4 cells. Thus, dilation and vesiculation of endoplasmic reticulum appear to be characteristics, as has been previously reported, of adenocarcinoma [11, 12] and malignant [8] cells.

These cell lines arise from adenocarcinomas by their morphologic characteristics, but the cell of origin of peripheral lung adenocarcinomas is a much debated issue, both in animal and human

tumors [1]. Typical type II alveolar epithelial cytosomes, composed largely of phospholipid, are not present in the HLAC-4 or HLAC-14 cell lines. Myelin figures, also phospholipid in nature [19], are quite common in these HLAC-4 and HLAC-14 cells, as well as in the tumor cells from which these cell lines were derived [1]. The difference between these myelin figures and cytosomes of type II alveolar epithelial cells in our tumors has been discussed [1]. Sommers *et al.* [20] saw laminar bodies (which we call myelin figures) in a line of transplantable human pulmonary adenocarcinoma cells. They concluded that these laminar bodies were different from cytosomes and that their tumor was of terminal bronchiolar origin.

Clara cells are considered the most likely cell of origin of our ^{210}Po-induced peripheral lung tumors [1], and have recently been implicated as the cell of origin of human peripheral lung tumors [21, 22]. However, neither Clara cells nor any other cell type in the normal hamster or human peripheral lung secretes mucus, and both adenocarcinomas of the peripheral lung and the original cheek pouch tumors grown from the HLAC-4 and HLAC-14 cell lines produce mucus. Our study of ^{210}Po-induced peripheral lung tumors suggests that Clara cells can be transformed into a mucus-secreting cell which is implicated in the progression of changes leading to malignancy [1]. Mucus production in the HLAC-4, HLAC-14, and SaBa [23] cell lines, all derived from the peripheral lung, ceases after continued passage. This is also the case for most other adenocarcinoma-derived cell lines [24], as well as for organ culture of normal lung [25]. However, mucus production continued in cell lines derived from adenocarcinomas of bronchial origin [23, 26]. This may be due to the different nature of mucus in the normal upper airway compared to the lower airway cells [27–29]. Whether mucus is actually produced by the cells in the HLAC-4 and HLAC-14 cell lines is not important for classification since they retain their adenomatous characteristics. The major cell types in cheek pouch tumors probably arise from the most prominent cell type of the primary lung tumor, a mucus-secreting adenomatous cell [1].

Various cell types are seen in cheek pouch tumors grown from cloned cell lines, each derived from a single parent cell. Such morphologic variation is common in epithelial tumors [30, 31]. HLAC-4 and HLAC-14 cells differ in their morphology, radiosensitivity and growth characteristics [2]. The epidermoid component of the original tumor remains in the transplanted HLAC-14 but not HLAC-4 cells. This may be related to differences in the original tumors, HLAC-14 cells having been derived from a tumor induced by combined ^{210}Po and benzo[a]pyrene treatment, and HLAC-4 from a peripheral lung tumor induced by ^{210}Po alone.

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Mammary Carcinogenesis in Castrated (C3H × RIII) F_1 Male Mice Bearing Ovarian Transplants in the Ear for Variable Periods of Time

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Abstract—*Increase in time of presence of an ovarian transplant on castrated male (C3H × RIII) F_1 mice resulted in a decrease in latency time and an increase in the frequencies of mammary tumours. A month long presence of an ovarian transplant was required to stimulate mammary carcinogenesis. The presence of an ovarian graft for either 2 months or throughout the life of the animal induced mammary tumours with a similar frequency. A 3-month graft, however, was necessary to obtain latency times similar to those observed in animals bearing life long grafts.*

INTRODUCTION

THE ROLE of the ovaries in mammary carcinogenesis was first pointed out by Lathrop and Loeb [1] as early as 1916. Murray [2] obtained mammary cancers in castrated male mice implanted with ovaries. Later, Lacassagne [3] showed that administration of oestrogens induced mammary tumours in male RIII mice. Fekete, Wooley and Little [4] observed that the appearance of mammary tumours in ovariectomized DBA mice was often accompanied by adrenal hyperplasia. They suggested that oestrogens of adrenal origin probably played a role in mammary carcinogenesis in these animals. The Berkeley team [5, 6] showed that in female C3H/Crgl mice, oestrogens were essential for mammary duct development, but neither for induction nor for maintenance of hyperplastic alveolar nodules and their transformation into mammary tumours.

Richardson [7] pointed out the advantage of (C3H × RIII) F_1 hybrids as an experimental model and showed that, in these animals, mammary tumour formation was stimulated by synthetic oestrogens [8].

We have used this experimental model [9] in our laboratory to test the effect of several gestagens, oestrogens and contraceptives on mammary carcinogenesis [10, 11]. Results obtained with hybrid females ovariectomized at different ages, provided evidence for the role of adrenal secretion of oestrogens in mammary carcinogenesis [12].

The aim of the experiments described here was to determine how time of presence of an ovarian graft in castrated males could modify mammary carcinogenesis in these animals.

MATERIAL AND METHODS

(C3H × RIII) F_1 hybrids were obtained by breeding C3H/Bi females and RIII males. Animals were maintained in a temperature (22°C ± 1°C) and light controlled (12 hr/day), room and provided a commercial diet (UAR 113b) and water *ad libitum*.

One-month-old hybrid males were castrated by the scrotal route. At the same time, an ovary just excised from a 4–5-week-old (C3H × RIII) F_1 female, was implanted under the skin of the dorsal face of the ear using a trocar.

Ovaries were removed by ablation of the part of the ear bearing the graft, fixed in Holland Bouin, stained with hemalun, eosin, saffron (H.E.S.) and examined systematically.

From the age of 2 months, the mice were examined once a week for palpable mammary tumours. The age of the mouse when the first

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tumour was detected was taken as the latency time.

Two control groups were used: one group (Te I) without ovarian graft and the second (Te II) with graft lasting the whole life-time. In the 6 experimental groups (E1 to E6), grafts were left in the ear for 10, 21, 30, 45, 60 and 90 days respectively.

Average latency times were compared using Student's *t*-test and frequencies using Pearson's χ^2 test.

RESULTS

Ovarian grafts

Out of 250 grafts performed, 4 were considered negative from the histology. These animals were discarded from statistical analysis.

Diameters of the excised grafts varied between 1 and 4 mm. The larger-sized grafts were observed especially during the 6-week-period after the grafts; they were congested. From 2 months on, the grafts became whitish; their size did not exceed 2 mm in diameter.

Histological appearance of the grafts varied as a function of time elapsed after the graft.

10-*day-post graft*

The ovaries were characterized by a dramatic hyperaemia and a vascular dilatation which disturbed the whole structure. Graafian follicles at all stages of maturation were observed.

21-*day-post graft*

The congestion was less important but a marked vascular dilatation persisted at the edge of the grafts and between the large follicles. They showed signs of atretic degeneration such as cystic dilatation, intrafollicular haemorrhage and compression of the granulosa cells. The follicles were surrounded by a dense fibrous *theca externa* (stroma) and interstitial cells. No corpora lutea were observed.

30-*day-post graft*

Vascular dilatation was still manifest around the grafts and near the ovarian hilus (Fig. 1A). Besides atretic follicles with blood-filled cavities, primary and secondary follicles were constantly observed. Corpora lutea-like structures appeared between the follicles at this time. They were often clearly delimited and consisted of several lobules of about 20 lutein cells (Fig. 1B).

90-*day-post graft*

The hyperaemia was greatly decreased. Many follicles at all stages of maturation and degeneration were present in the grafts (Fig. 1C). Blood was very rarely present in the follicular cavities but cystic degeneration was frequent. The degenerative follicles reached much bigger sizes than mature follicles *in situ* and were often delimited by fibrous stroma. Well-defined corpora lutea were constantly found (Fig. 1D).

The following stages of evolution of the grafted ovaries could be observed in animals bearing a graft for their whole life time. In several animals sacrificed 5–6 months after the graft, the ovaries showed the first signs of sclerosis and even of fatty degeneration. Nevertheless, in the majority of cases, the 6-month-old grafts were hardly different from those removed 2 or 3 months after the graft. One-year-old grafts showed a proliferation of the fibrous stroma, the presence of anovular follicles, and a decrease in the graft volume. All ovaries removed more than $1\frac{1}{2}$ yr after the graft were sclerotic.

Mammary carcinogenesis

Frequencies and average latency times of the mammary tumours for the various experimental groups are listed in Table 1. The distribution of latencies are represented by the cumulative curves of frequency of Fig. 2.

In animals bearing a 1-month-graft, there was a significant ($P < 0.02$) increase in the frequency of mammary tumours as compared to the control group Te I. The frequency of tumours in animals bearing a 2-month-graft was about the same as that in animals bearing a graft for their life-time.

The average latency was inversely proportional to the duration of the ovarian graft. An increase in time of presence (1–3 months) resulted in a progressive decrease of the average latency. However, latencies observed were the same whether animals had a 3-month- or a life-long graft.

DISCUSSION

We have studied the effect of the time of presence of an ovarian graft on mammary carcinogenesis in castrated male (C3H × RIII) F_1 mice. We have shown that a short-time graft (10–21 days) had no significant effect as regards either the frequency or the latency time of the tumours. On the contrary, 2–3 month-long grafts resulted in a stimulation of carcinogenesis, ranging about that obtained in animals with a life-long graft.

Richardson [13] reported that the frequency of mammary tumours in virgin (C3H × RIII) F_1 female mice was significantly reduced when ovariectomy was performed at 4 months (i.e., functional ovaries were present for 3 months), the frequency of mammary tumours was not

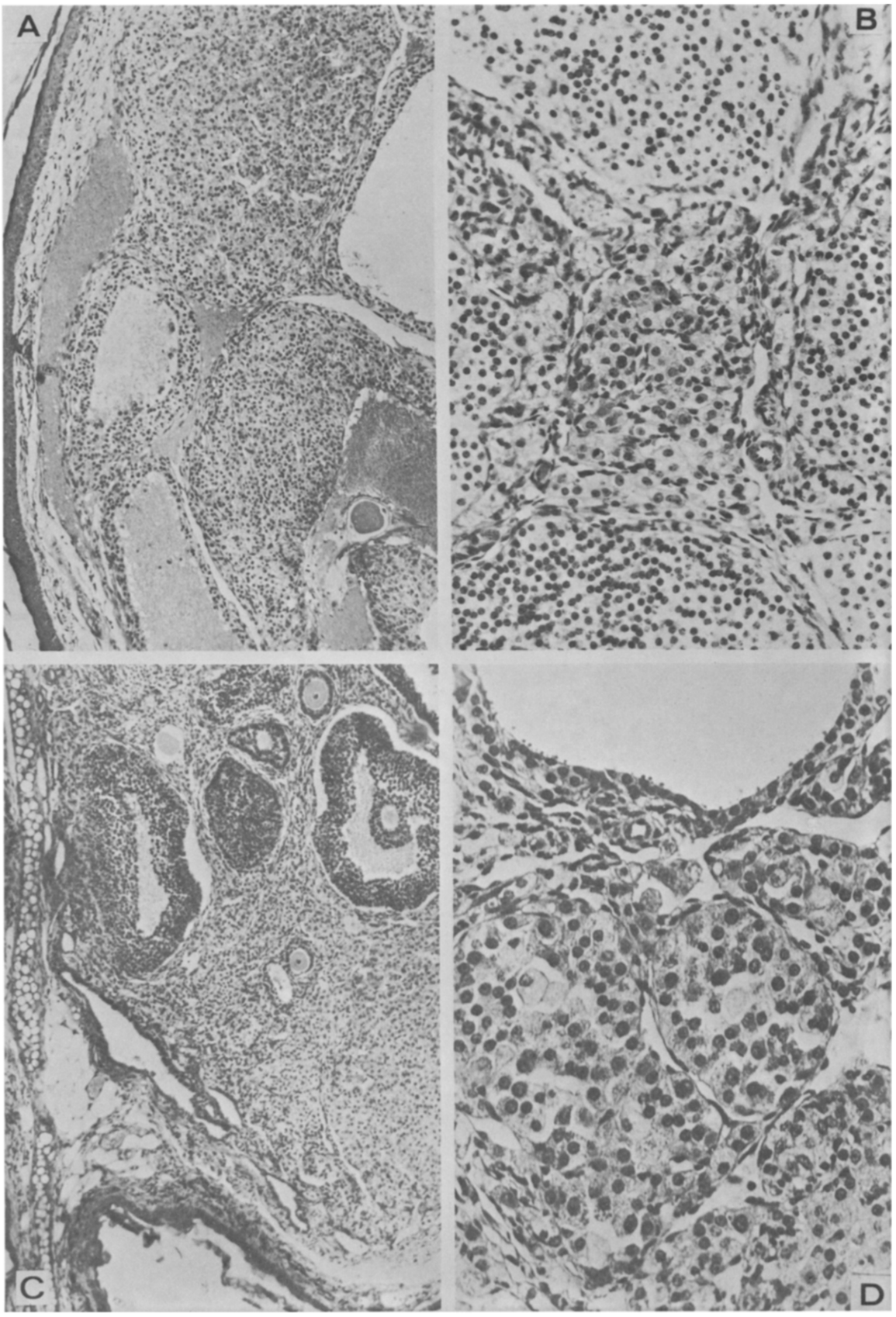

Fig. 1. Histological sections of the ovarian grafts after variable time of presence in the ear of castrated (C3H × RIII) F_1 male mice.
(A) 30 days post graft. Vascular dilatation around the graft and the cystic dilatation of the follicular cavity with compression of the granulosa cells in top right corner (H.E.S. × 75).
(B) 30 days post graft. Corpora lutea in formation (H.E.S. × 200).
(C) 90 days post graft. Functional ovarian graft with follicles at variable stages of the maturation (H.E.S. × 75).
(D) 90 days post graft. Same ovary as that in C: enlargement of the well defined corpora lutea (H.E.S. × 200).

Table 1. Mammary carcinogenesis in castrated (C3H × RIII) F_1 male mice bearing ovarian transplants in the ear for variable periods of time

Treatments	No. of animals	Animals with mammary tumour No.	%	Tumour latency Mean ± S.D. (days)
Castrated males (Te I)	28	3	10	602 ± 13
Castrated males bearing the ovarian graft during				
10 days (E 1)	10	1	10	250
21 days (E 2)	24	6	25	512 ± 40*
30 days (E 3)	30	12†	40	411 ± 21‡
45 days (E 4)	43	19§	44	323 ± 20‖
60 days (E 5)	36	34	94	235 ± 14¶
90 days (E 6)	41	29	71	192 ± 10
Life-time (Te II)	26	22	85	198 ± 14

*Significantly different from E 3 ($P<0.05$).
†Significantly different from Te I ($P<0.02$), Te II ($P<0.001$), E 6 ($P<0.01$).
‡Significantly different from Te I ($P<0.001$), E 4 ($P<0.01$).
§Significantly different from Te I ($P<0.01$), Te II ($P<0.001$), E 6 ($P<0.02$).
‖Significantly different from E 5 ($P<0.01$).
¶Significantly different from E 6 (0.01).

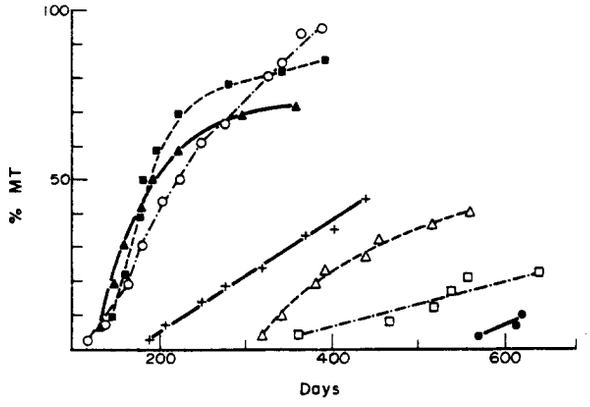

Fig. 2. Effect of the time of presence of ovarian graft on the frequency of palpable mammary tumor (MT) in castrated (C3H × RIII) F_1 male mice.
The time of presence of ovarian graft was respectively: ■----■ *life time,* ▲——▲ *90 days,* ○·—·—·○ *60 days,* +——+ *45 days,* △----△ *30 days,* □·—·—·□ *21 days,* ●——● *animals without ovarian graft.*

different from the one observed for intact females. Our results on the frequency of mammary tumours in castrated males bearing a 2 month graft (tumours in nearly all the animals) agree with this report [13]. The frequency of mammary tumours obtained in males bearing a 4–6-week-graft also agrees with the frequencies observed for ovariectomies performed at 2–3 months of age [13].

Our results for the latencies seem to indicate that the presence of the ovarian graft had no effect on the tumour growth during the stages just before the appearance of a palpable tumour. Castrated males bearing ovarian grafts for 2–3 months could be used as an experimental system for investigating the role and/or mechanisms of action of ovarian secretions on mammary carcinogenesis.

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Letter to the Editor

Influence of Intravenously Injected ^{131}I-Labelled Albumin on the Growth Rate of a Sarcoma Transplanted into the Rat Stomach

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A HIGH permeability of the tumour capillary wall for large protein molecules and a temporary retention of labelled albumin and fibrinogen was found in transplantable rat tumours [1–3]. A high radioactivity was also recorded in normal rat stomach after intravenous (i.v.) injection of labelled albumin and was explained by albumin being eliminated in the stomach and upper small intestine [4]. These observations stimulated a study on the influence of intravenously injected, heavily ^{131}I-labelled albumin by local isotope irradiation on the growth rate of a 20-methylcholanthrene induced sarcoma transplanted into the rat stomach.

Tumours were transplanted under ether anaesthesia into the stomach of inbred Lister rats with a mean body weight of 200 g, according to a method described by Brøyn [5]. Through a small upper transverse abdominal incision, the rat stomach was lifted up into the wound. A polyethylene catheter was passed through the mouth into the stomach. A concentrated mechanically produced tumour cell suspension (1 ml) was injected into the stomach and the stomach wall was traumatized by an arterial clamp over an area of 10 mm^2. The stomach tube was withdrawn under aspiration and the abdominal wall was closed with two layers of sutures.

Two weeks after tumour transplantation, small tumours with a diameter of 3–5 mm were palpable in the traumatized stomach wall in 100% of the animals during a new laparotomy. These tumours grew and killed the animals within 3–4 weeks. The general condition of the animals was not influenced by repeated laparotomies. However, animals were sacrificed when their general condition was influenced by large tumours. Metastases to liver and lungs were found in 10% of the animals.

The tumour growth rate was recorded at repeated laparotomies by measuring 3 dia (a, b and c mm dia) on day 0 (2 weeks after transplantation), 5, 12 and 21 of the experiment. The tumour volumes were calculated from these diameters according to the formula for a spherical body:

$$\left(\frac{a}{2}\times\frac{b}{2}\times\frac{c}{2}\right)\times\frac{4\pi}{3}=\text{tumour volume in mm}^3.$$

Forty-five rats were transplanted with tumour 2 weeks before the start of the experiment. Fifteen animals were untreated controls, 15 animals were injected i.v. with a single dose of 1 mCi of a heavily ^{131}I-labelled human albumin (Kabi Diagnostica, Sweden) with a specific activity of 2.3 mCi/mg albumin, and 15 animals were injected with 0.35 mCi of labelled albumin 4 times with 2 days interval, starting on day 0. The tumour growth was followed in all groups and mean values are presented in Fig. 1. Control animals and animals given a single dose of labelled albumin were sacrificed on day 12 of the experiment due to large tumours, while animals given repeated injections could be followed until day 21.

A single injection of 1 mCi of labelled albumin did not significantly influence the tumour growth rate, which was, however, significantly

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retarded by repeated injections of labelled albumin, as could be seen on day 12 of the experiment. This retardation was temporary and large tumours were found in this group of animals at sacrifice on day 21.

A high tumour capillary permeability for large protein molecules suggests that radioactive substances bound to large protein molecules might be concentrated in tumours and used in diagnosis and therapy. However, this mechanism is limited by a passive plasma–tissue equilibration of proteins in tumours [3] and a sufficient therapeutic concentration of the active substance in tumour tissue is hard to reach in spite of administered high doses of isotope. This was observed in the present study, in which tumours were also transplanted into the stomach to reach a high radioactivity in normal tissue surrounding the infiltrating tumour, by labelled albumin being eliminated in this organ.

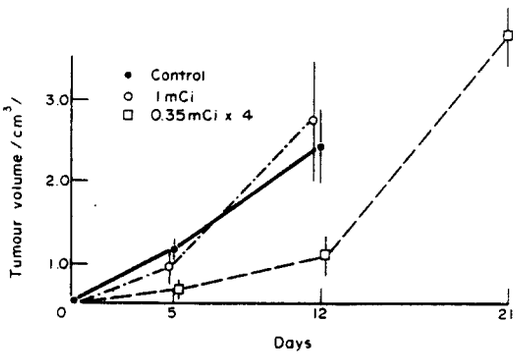

Fig. 1. Mean volumes (with S.E.M.) of gastric tumours in 15 control rats, 15 rats given a single i.v. dose of 1 mCi ^{131}I-labelled human albumin on day 0 and 15 rats given 0.35 mCi ^{131}I-labelled albumin 4 times with 2 days interval, starting on day 0. A significant ($P < 0.05$) but temporary reduction of the tumour growth rate was found in animals given repeated doses of labelled albumin on day 12.

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Perspectives in Cancer Research

An Overlooked Aspect of the Mechanism of Action of Most Antineoplastic Drugs: The Inhibition of Macromolecular RNA Metabolism

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IN THE majority of modern textbooks of hematology or clinical oncology, and sometimes in specialised papers also, the problem of the mechanism of action of almost all the cytostatic drugs is rapidly dismissed by referring to their ability to inhibit DNA synthesis. This appears, in view of the rapidly growing knowledge of RNA metabolism in animal cells, a gross oversimplification. As a matter of fact, some observations suggest that the very possibility that some drugs affect differentially normal and malignant cells rests on their ability to affect RNA macromolecular metabolism rather than DNA synthesis.

About 20 yr ago, radioautographic studies [1] had already shown a fact of basic relevance to the problem which will be discussed in this paper.

Whereas the activity of the DNA-directed DNA polymerases in a leukemic blast cell population occurs in a fraction of the whole cell population which is smaller than the comparable fraction of normal bone marrow precursor cells, a completely different behavior has been observed for the DNA-directed RNA polymerases. These enzymes display their activity in all blast cells, no matter they are in or out of cycle, and throughout the entire interphase.

This observation, in view of the evidence which will be reviewed beyond, clearly indicates that all leukemic cells, and not only DNA synthesizing cells, are the target for most cytostatic drugs.

MAIN FEATURES OF RNA MACROMOLECULAR METABOLISM

The really critical progress in this area is represented by the elucidation of the structural and metabolic characteristics of the primary product of the activity of the RNA polymerases. In fact, it has been shown that neither the 2 RNA components of the ribosomal subunits, 18S and 28S RNA, nor the messenger RNAs, are synthesized in their final forms. On the contrary, they are synthesized as much larger "precursors" which must undergo extensive "processing" to reach a condition of functional "maturity". As shown by Sherrer and Darnell several years ago [2, 3], the ribosomal RNA precursor is a very large molecule having a well definite molecular size, corresponding to an S value of 45. In terms of mol. wt, it is about 4.5×10^6 daltons. An electron microscopy picture of this giant molecule has been reported by Wellauer and Dawid [4]; it shows clearly, interspersed along the molecule, "loops", segments with secondary structure, which might have a critical role in the cleavage process [5].

The conversion of this giant molecule to its lower mol. wt final products follows a well definite pattern. 45S is cleaved into 41S and another large fragment, which is degraded. 41S gives rise to 32S and 20S, and these in turn are cleaved to 28S and 18S.

The precursors of messenger RNAs are represented by the so-called heterogeneous nuclear RNA [6, 7] an heterogeneous population of molecules ranging in size from 20S up to 100S. However, according to some authors [8], the "nascent" messenger precursor molecules are only those molecules larger than the 45S, which represent about 55% of the entire heterogeneous nuclear RNA. The smaller molecules should represent only intermediate products of the

cleavage process, which leads to messenger molecules of size not larger than 12–18S, leaving about 90% of the initial molecule to degradation by nucleases. No definite pattern of processing has been so far demonstrated for messenger precursors. The rates of processing of the ribosomal and messagerial precursors, and of consequence the life-span of these molecules, are largely different according to the functional state of the cell. In rapidly synthesizing cells, such as PHA-stimulated lymphocytes, the average life of the 45S ribosomal precursor, or of the giant messenger precursor RNA is only a few minutes. A quick indication of this fact is obtained by studying the distribution of radioactivity in fractions of whole cell RNA of different size after incubation of PHA-stimulated lymphocytes for 30–60 min with a radioactive RNA precursor. The large majority of the label is associated with RNA molecules smaller than 30S, and mainly with the 28S and 18S ribosomal RNA [9]. A markedly different pattern is obtained when we incubate with [5^3-H] uridine resting, out of cycle cells, such as small circulating lymphocytes. In this case, even after 8–12 hr of incubation, the majority of radioactivity is still associated with molecules larger than 30S [9].

Mechanisms controlling the rate of RNA processing in eukariotes are at present very poorly understood. Only in the last few yr the possibility has been explored that a group of specific RNases carry out highly complex reactions.

However, as pointed out recently by Robertson and Dickson [10], we must accept the idea that control by RNA processing should be considered coequal with control of transcription or translation as an important way in which cells regulate their expression.

ACTION OF CYTOSTATIC DRUGS ON MACROMOLECULAR RNA METABOLISM

It is commonly considered that RNA synthesis can be inhibited by drugs acting at 4 different levels: (1) by blocking *de novo* synthesis of purines and pyrimidines, or inhibiting the synthesis of nucleotides, thus acting at the level of the substrate; (2) by inactivating DNA, thus acting at the level of the template; (3) by directly affecting the catalytic activity of the enzyme, thus acting at the level of the enzyme; (4) by inhibiting the processing of the immediate products of transcription, causing accumulation of unprocessed molecules, thus acting at the level of the precursors.

Two clinically important drugs, whose antitumor effect is usually referred to their inhibitory activity on DNA synthesis, affect RNA metabolism through one of the first three mechanisms above mentioned. The first one is cytosine arabinoside (ara-C), which is currently one of the most successful drugs used to induce remission in acute myeloid leukemia. Its cytotoxic activity is universally ascribed to its ability to inhibit DNA polymerase after being phosphorilated intracellularly to its triphosphate derivative (araCTP) [11]. Studies with various cell lines have however shown that acute cell death induced by ara-C does not correlate well with the inhibition of DNA synthesis [12]. As a matter of fact, recent studies have shown that araCTP is a competitive inhibitor for the binding of CTP to RNA polymerase II, and that the affinities of ara-C for the RNA polymerase and the DNA polymerase are comparable [13]. These studies suggest the possibility that RNA polymerase is a target for ara-C action, so that inhibition of RNA synthesis must be considered to adequately understand the action of this drug.

Another well known drug whose action is universally referred to inhibition of DNA synthesis is the antifolate methotrexate. By inhibiting dihydrofolate reductase MTX causes a depletion of intracellular pools of reduced folates, which blocks many reactions, most critical of which may be the conversion of deoxyuridilate to thymidilate. However, it has been observed that addition of hypoxanthine to the culture medium as an exogenous source of purines considerably prevents the cytocidal effect of MTX. This suggests that MTX induces a "purineless" death as well as a "thymineless" death [14], and the mechanism of purineless death may be related to the suppression of RNA synthesis. MTX suppresses [5^3-H] uridine incorporation into RNA to a many fold greater degree in faster proliferating cells than in resting cells. In the two instances just mentioned, the effect on RNA metabolism may not be as important quantitatively as the effect on DNA synthesis. However, there is enough evidence to make difficult to assign the antitumor activity of the two drugs only to their ability to inhibit DNA synthesis. But far more interesting appear the results of the study of the action on RNA metabolism of almost all the other commonly used cytostatic drugs. These results indicate in fact that RNA synthesis is mainly inhibited by acting at the fourth level, i.e., at the level of processing of the precursors. Let us briefly examine the mechanism of action of some clinically important alkylating agents. Two well known and extensively used drugs of this class

have been studied in our and other laboratories, mechloretamine (HN_2) and 1,3-bis(2-chloroethyl) 1 nitrosourea (BCNU) [15, 16, 17]. We have shown some years ago [15] that exposure of PHA-stimulated lymphocytes to nitrogen mustard leads to inhibition of 45S RNA synthesis, possibly because of a block in the processing of the precursors, evidenced by accumulation of radioactivity in 45S and 32S, and by inhibition of methylation. A similar effect has been observed [16, 17] by studying the action of BCNU, which also strongly inhibits nucleolar processing of the 45S and 32S RNA, thus leading to decreased RNA synthesis. Both substances affect in some way the synthesis and processing of messenger precursors, since the appearance of cytoplasmic poly(A)-containing messenger RNA is completely inhibited [16]. CCNU also inhibits synthesis and processing of 45S RNA [17].

It must be pointed out that the effects of these three alkylating agents on macromolecular RNA metabolism appear to be relatively specific, and are not the result of a general deterioration in cellular metabolism following administration of the drugs. In particular, the action of these drugs, for several reasons, does not appear to result from any possible effect on DNA.

Let us examine the effects on RNA metabolism of another large family of antineoplastic drugs, the base analogs.

All purine and pyrimidine analogs studied so far have been found to inhibit processing and maturation of ribosomal precursor RNA [18–22]. Incorporation of the analogs into RNA is the most likely explanation of their ability to inhibit the maturation process. Such incorporation should alter the structure of the precursor RNA and should not allow normal processing.

It has to be emphasized that analogs of each natural base, i.e. cytidine analogs [18–20], uridine analogs [21], guanine analogs [19] and adenosine analogs [22] have been found to cause inhibition of ribosomal RNA maturation.

The peculiar activity on RNA metabolism of two further drugs whose action has been studied in the author's laboratory deserves attention. The first one is Daunorubicin, the glycosidic anthracycline which has shown high efficacy in the treatment of human leukemia and is widely used in several different antileukemic chemotherapy schedules. Daunorubicin is notoriously a strong inhibitor of both DNA and RNA synthesis. Exposure of normal proliferating lymphocytes to this antibiotic brings about an extremely rapid and complete inhibition of the appearance of 18S and 28S mature ribosomal subunits, and accumulation of 32S and 45S RNA is observed [15]. Although no definite explanation may be offered, it is worth to mention that it has been shown that, not unlike what happens with DNA, daunorubicin is able to form a complex with double-helical RNA [23]. As already mentioned, double-helical segments seem to have a critical role in processing of large ribosomal and messagerial precursor RNA molecules.

Even more interesting is the action shown on the processing of RNA precursor molecules by the vinca alkaloids. Vincristine sulfate is a well known mitotic poison, but it is less known that it also markedly affects macromolecular RNA metabolism. In the presence of vincristine the formation of 18S and 28S ribosomal RNA components is almost completely inhibited and 45S and 32S ribosomal precursors accumulate [15]. As observed by Wagner and Roizman [24] the same effect is shown by vinblastine sulfate, the other vinca alkaloid commonly used. So far, no explanation may be presented for this peculiar activity of these alkaloids.

Also in these cases, however, the effect of the drugs appears to be highly specific and is not the result of protein synthesis inhibition or of a general deterioration in cellular metabolism following the administration of the drugs.

RELEVANCE OF THE ACTION ON MACROMOLECULAR RNA METABOLISM TO THE CELLULAR PHARMACOLOGY OF ANTITUMOR DRUGS

The very fact that many drugs of several different classes far from each other, such as alkylating agents, base analogs, antibiotics and alkaloids, all of which are considerably clinically effective in treating malignancies, appear able to inhibit RNA synthesis by inhibiting processing of the precursor RNA molecules, seems to indicate that this effect is at least in part responsible for the antitumor activity of these molecules. Some basic consideration of cell physiology will lend support to this hypothesis. In rapidly dividing cells, ribosomal RNA synthesis goes on at a very high rate. It has been calculated that each ribosomal RNA cistron (and they have been estimated at more than one hundred), would have to be transcribed 20 times per min continuously during the 24 hr generation time to produce the average cell complement of ribosomal RNA [25, 26]. This means that the conversion of the high molecular weight

precursor of ribosomal RNA to its lower final products must go on very fast. This makes the rapidly proliferating cells very sensitive to the action of substances able to impair processing of large RNA molecules.

In resting cells, the demand for new ribosomal RNA synthesis is much less. This suggests that inhibition of ribosomal RNA maturation may be useful in selectively destroying a population of rapidly dividing cells. The effect of many antitumor drugs on RNA metabolism might thus help as well as the effect on DNA synthesis, to explain the observation that, generally speaking, proliferating cells are much more sensitive to anticancer agents than are non proliferating cells [27].

On the other hand, the ability to impair preribosomal RNA processing may also help to explain the effectiveness of several drugs in reducing rapidly the leukemic cell populations, which consist predominantly of cells in a more or less quiescent state.

Some light on this effect has been shed by the results of the study of the metabolism of nuclear RNA precursor molecules in leukemic blast cells. In these cells, the overall rate of synthesis of cellular RNA, 80% of which is represented by ribosomal RNA, is low, although this observation does not fit with the existence of highly developed nucleoli in these cells. These structures are in fact the site of synthesis and processing of preribosomal RNA. An explanation may be seen in the observation we made some years ago, that the life of 45S and 32S ribosomal precursor RNA of acute leukemia cells is much longer than that of rapidly synthesizing cells, such as PHA-stimulated lymphocytes [28, 29].

Further studies have shown that giant heterogeneous RNA molecules larger than 45S are processed at an even lower rate, so that they accumulate in leukemic blast cells [30].

Under these conditions, whatever may be the cause of the slow processing of the ribosomal and messagerial precursors, a further marked decrease in the rate of final RNA molecules "maturation", originated by exposure to a sufficiently high level of drug, must be severely detrimental to the cell, bringing to premature cell death. It has been recently pointed out [27] that, at this time, many experimental and clinical oncologists consider that the physiology and drug sensitivity of all out of cycle cells are the same, no matter they are normal or neoplastic, and base much of their conceptualization on the resting hemopoietic stem cell. Were the partial failure of the leukemic cells to convert the high mol. wt precursors to their final products to keep them "out of cycle", this might represent a major difference from the hemopoietic G_0 stem cells, whose quiescent state is presumably maintained through different mechanisms.

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Use of Glycoprotein Antigen in the Immunodiffusion Test for Bovine Leukemia Virus Antibodies

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Abstract—*The agar gel immunodiffusion (AGID) test for detection of bovine leukemia virus (BLV) antibody was modified by substitution of glycoprotein antigen for p24, the ether-stable, internal viral antigen used in earlier investigations. This modified AGID test was compared with a complement fixation (CF) test that had shown superior sensitivity to the first AGID procedure.*

Sera from 263 cattle in commercial dairy herds were tested. The glycoprotein AGID test detected BLV antibodies in 171 sera, but the CF test detected only 134. In monthly examinations of sera from animals in a BLV experimental herd, the AGID test consistently detected more infected cattle. After experimental or natural infection, 14 of 19 cattle seroconverted to both the AGID and CF tests the same month. When antibodies were detected by only one test, it was usually the AGID test. Colostral antibodies in 2 calves from BLV-infected dams were detected longer by the AGID test than by the CF.

These results show that the glycoprotein AGID test is more sensitive than the CF test for detection of BLV antibodies in cattle sera. The AGID test also offers distinct advantages in its simplicity and specificity and promises to be a useful tool for the development of BLV control and eradication programs.

INTRODUCTION

In 1972, Miller and Olson [1] described an agar gel immunodiffusion (AGID) test that could be used to detect antibodies to bovine leukemia virus (BLV). The test was designed to identify antibodies to an antigen (p24) obtained by disruption of purified virus particles with ether. Several investigators used this AGID test in serologic studies that confirmed the association of BLV and bovine enzootic leukosis [2–6]. However, subsequent comparative studies with other serologic tests (complement fixation and indirect immunofluorescence) clearly showed that some BLV-infected cattle were not detected by the AGID test [7–9].

Recently, the finding of a second precipitating antigen associated with BLV was reported [10, 11]. The antigen was shown to be a glycoprotein and treatment with ether resulted in loss of antigenic activity. A procedure was devised for concentration and isolation of the BLV glycoprotein from fluids of persistently infected monolayer cell cultures, and the antigen recovered by this procedure was used to develop an improved AGID test for BLV antibody [11]. This paper presents a comparison of the new AGID test with the CF test described previously by us [12].

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MATERIAL AND METHODS

Cell cultures

Fetal lamb kidney cells persistently infected with BLV were the source of antigens used in this study. The development and maintenance of similar ovine cell lines have been described elsewhere [13, 14]. Fluids were collected weekly at the time cells were subcultured.

Antigens

Culture fluid was centrifuged at 1000 ***g*** for 30 min to remove cell debris and used without further treatment as antigen for the CF test. Glycoprotein antigen for the AGID test was precipitated from culture fluid by the addition of crystalline ammonium sulfate at the rate of 30 g per 100 ml. The salt was dissolved with the aid of a magnetic stirring device and then the mixture was allowed to stand at 4°C for 72 hr. At that time, the supernatant fluid was removed by aspiration and the precipitate was further concentrated by centrifugation at 7000 ***g*** for

15 min. The precipitate was resuspended in phosphate-buffered saline (PBS) to approximately one-tenth the original volume. The suspension was held overnight at 4°C and precipitate which formed during this holding period was removed by centrifugation at 1000 *g* for 30 min. The supernatant fluid was collected and passed through a column containing Con A-Sepharose (Pharmacia Fine Chemicals. Uppsala, Sweden). The column was then washed with PBS and the BLV glycoprotein was eluted with 0.1 M alpha-methyl-D-mannoside. Eluate fractions were tested for antigen content by AGID with BLV antiserum from an experimentally infected cow. Positive fractions were pooled and then concentrated or diluted as necessary to give a sharp line in AGID with an appropriate dilution of antiserum. The desired combination gave a precipitation line that was sharp, bright and long enough so that the line continued into adjacent wells that contained negative serum (Fig. 1). No preservative was added to the antigen and it was frozen when not in use. Specificity of the antigen for BLV antibody was tested with antisera to the following bovine viruses: infectious bovine rhinotracheitis, bovine virus diarrhea, bovine syncytial virus, bovine maedi-like virus, parainfluenza 3 and bovine respiratory syncytial virus.

AGID test

Test plates for AGID were prepared with 0.8% agarose (SeaKem, Marine Colloids, Inc., Rockland, ME) in 0.05 M Tris-HCl buffer, pH 7.2, which contained 8.5% sodium chloride. The agarose was distributed into 60-mm plastic Petri dishes; a vol of 6 ml per dish was used. The standard test pattern consisted of 1 central and 6 peripheral wells, each 8 mm dia, with a 2.5 mm distance between reactant wells (Fig. 1). The appropriate dilution of positive control serum was placed in 3 peripheral wells alternately with 3 undiluted test serums and the center well was filled with antigen. Test plates were held at room temperature in a humidified chamber and examined after 48 hr. Serums were recorded as positive when they formed lines of identity with the control serum or when they caused the ends of the control lines to "bend" inward toward the antigen well (Fig. 1).

CF test

A microtiter system (Cooke Engineering Co., Alexandria, VA) was used to make twofold dilutions of the test sera in veronal buffer. The serum dilutions were prepared in 25 μl vol and 25 μl of antigen was added to each well. Anticomplementary activity of each serum was also tested at dilutions of 1:2 and 1:4 by substitution of veronal buffer for antigen. Guinea pig serum was the source of complement. As described previously [12], it was supplemented with unheated normal bovine serum to a final concentration of 5%. The complement was diluted to give two, 100% hemolytic units in 25 μl of the final preparation. The test plates were incubated at 4°C for 16–20 hr, at which time sensitized sheep red blood cells were added. The sensitized cells were prepared by mixing equal vol of rabbit hemolysin and a red cell suspension that had been adjusted photometrically to give an optical density of 0.25 at 550 μm after lysis of the red cells in distilled water. The hemolysin-red cell mixture was left at room temperature for 20 min: then 25 μl was placed in all test and control wells. The plates were incubated at 37°C for 1 hr and examined visually to determine the titration endpoint of 50% hemolysis. Only serums with titers of 1:4 or greater in the absence of anticomplementary activity were considered positive.

Test sera

For an earlier report that compared the 1972 AGID test with the CF test [7], we used sera from cattle in commercial dairy herds known to have BLV-infected animals. These same sera were used in the present study to assess the relative sensitivity of the new AGID test. A second group of sera was available from cattle used in various BLV transmission experiments. These sera have been collected on a monthly basis and include samples from cattle with natural, as well as experimentally induced, infections.

RESULTS

Results of the CF and the new AGID test on sera from cattle in commercial dairy herds are given in Table 1. There was agreement between the 2 tests in 84% (222 of 263) of the sera examined, and more than half of these were in the positive category. The major discrepancy resulted from sera that were positive in AGID but negative in the CF test (39 sera). Only 2 sera gave positive CF reactions and negative AGID results. The CF titers of these samples were relatively low, 1:4 and 1:8, and neither serum had reacted in the old AGID test [1] with ether-treated antigen.

Comparison of the CF and AGID tests on sera taken from cattle in the BLV experimental herd are given in Table 2. At each sampling time, more sera were positive by the AGID test than by CF. When there was lack of agreement between the 2 tests, it was always due to a serum that gave a positive AGID reaction but was negative in the CF test.

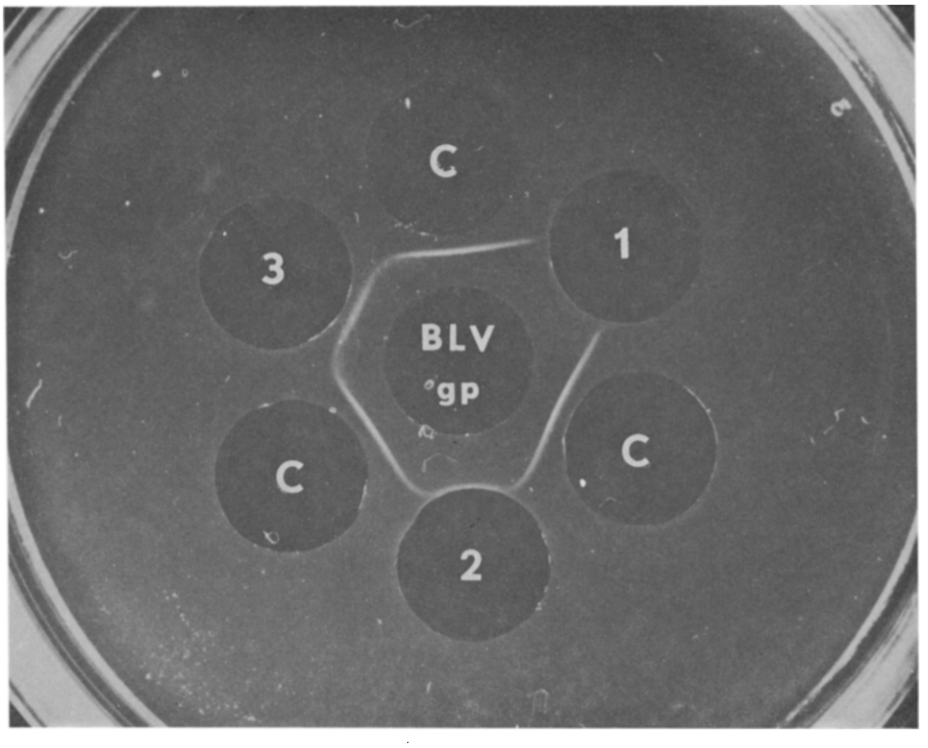

Fig. 1. Agar gel immunodiffusion test for detection of antibodies to BLV. Center well: BLV glycoprotein antigen. Peripheral wells: C—control positive serum from an experimentally infected cow; 1—serum from a cow in a BLV-free herd; 2 and 3—sera from 2 cows with naturally acquired BLV infections. Serum in well 2 is considered a weak positive because it only "bends" the ends of lines formed by the control serum.

Table 1. Comparison of glycoprotein AGID and CF tests for serologic detection of BLV infection in 263 cattle from commercial dairy herds

Result		No. of sera	Total examined (%)
AGID	CF		
+	+	132	50
−	−	90	34
			84% agreement
+	−	39	15
−	+	2	<1
			15% non-agreement

Table 2. Comparison of glycoprotein AGID and CF tests for detection of BLV-infected cattle in experimental herd

Month	No. tested	No. AGID+	No. CF+
Nov., 1975	50	40	37
Dec., 1975	50	40	39
Jan., 1976	52	39	35
Feb., 1976	52	40	38

Monthly serum samples from 19 cattle with experimentally induced or naturally acquired BLV infections were examined to determine the relative sensitivity of the AGID and CF tests in detection of early antibody responses. These results are summarized in Table 3. In most of the cattle, seroconversions were detected the same month by both tests. Only 1 animal had a detectable CF response before precipitating antibody appeared and in this serum the CF titer was only 1:4. In 4 animals, AGID reactions were positive before CF antibody was detected. With one exception, when the first positive reaction was detected by only 1 of the serologic tests, the other test was also positive the next month. The exception was a calf which developed BLV infection as a result of contact exposure. The AGID reaction of this animal was positive for 2 consecutive months before seroconversion was detected by CF.

Another comparison of the AGID and CF tests involved the monthly examination of sera from 2 calves of BLV positive dams. Precolostral sera were negative for antibody, and after colostrum feeding, both calves were seropositive by AGID and CF. In one calf, passive CF antibody persisted for only 1 month. The calf then remained seronegative until CF antibody was again detected at 6 months of age. During this 5-month interim, the serum samples continued to give positive reactions by the AGID test, although they gradually became weaker. At the time CF activity reappeared, the AGID response was again very strong. This animal then remained seropositive by both tests during the next 18 months of observation. The second calf

Table 3. Comparison of glycoprotein AGID and CF tests for detection of early serologic response to BLV infection (monthly examinations)

Type of exposure	No. of animals examined	Antibody first detected by:		Simultaneous seroconversion
		AGID	CF	
Experimental	12	3*	0	9
Natural	7	1	1	5

*No. of animals.

had a continuous but decreasing CF titer to BLV for 7 months and a positive AGID reaction for 10 months. After becoming seronegative at 11 months of age, the animal has remained negative through the next 13 months of observation.

DISCUSSION

The AGID test with glycoprotein antigen was shown by these studies to be substantially more sensitive than the CF test for detection of BLV antibodies in cattle sera. In the study of commercial dairy herds, 171 sera were determined positive by AGID but only 134 sera gave positive reactions in the CF test. A similar finding was reported in a more limited study involving sera submitted to a federal diagnostic laboratory. Of 16 sera that gave positive AGID reactions, only 13 had CF antibodies [15]. Note that although these results suggest that it is relatively common for cattle infected with BLV to have precipitating but not complement fixing antibodies, we cannot be certain that the AGID and CF tests measure antibodies to the same antigens. The antigen used in the AGID test described here has been isolated and characterized [16], but the antigen or antigens in culture fluid that react in the CF test have not been identified.

The AGID test was more consistent than the CF test in detecting BLV reactor cattle in our experimental herd although the difference in sensitivity was not as great as that observed in the commercial herds. This result might have been anticipated because experimentally infected animals were probably exposed to a larger dose of virus than those infected under natural conditions and could therefore be expected to develop higher antibody titers. Some of the test discrepancies involved sera from recently infected animals, and as the monthly serum tests indicated, seroconversions to BLV were often detected by AGID while the CF test was still negative. Furthermore, we have observed in our monthly serologic tests that a few cattle consistently have AGID-positive but CF-negative reactions. These animals have usually been infected for a long time and their CF titers during the early stages of infection were comparatively low.

The 2 sera that gave positive CF but negative AGID results in the commercial herd study may have resulted from non-specific reactions. We check the specificity of each lot of CF antigen by testing 24 sera from cattle in a BLV-negative herd, but these tests may not be adequate to accommodate the individual variability encountered when large numbers of sera are tested. In this respect, the AGID test has a distinct advantage over CF because a test serum that produces a line of identity with the positive control serum (or one that "bends" the control serum line) has satisfied the requirement of specificity.

The examination of sera from 2 calves of BLV-infected cows showed that the AGID test was also a more sensitive indicator system for detection of passive antibody than was the CF test. However, the CF test did offer the advantage of providing antibody titers. Using this information, we were able to follow the decrease of passive antibody levels and then observe in 1 calf a subsequent increase of titer, presumably due to an active BLV infection.

We have concluded from our investigations that the glycoprotein AGID test for BLV antibody is superior to the CF test in respect to sensitivity, simplicity and specificity. Straub has reported that AGID tests corresponded very well with hematological data in a study on experimentally-infected cattle [17]. Further comparisons with the hematologic test and with other serologic tests will be necessary to completely evaluate the glycoprotein AGID test for BLV.

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Fine Structural, G-6-PD Isoenzyme, and HaLV *gs* Antigen Studies of Poly I/C and Antiestrogen Treated DES-Induced Hamster Renal Tumors*

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Abstract—*Poly I/C an interferon inducer with secondary antiviral action and antiestrogens ICI* 46,474 *and U*-11,100*A were given to hamsters carrying DES-induced and dependent renal tumors. Both promoted a reversal in the G*-6-*PD isoenzyme pattern to that pattern observed in non-tumours adjacent kidney tissue and normal non DES-treated kidney. Poly I/C reduced the expression of HaLV* gs *antigen. Cytological changes in the DES-dependent tumors resulted from treatment by Poly I/C and antiestrogens. Growth of DES-independent tumors was not inhibited following Poly I/C treatment.*

INTRODUCTION

FINE structural characteristics, glucose-6-phosphate dehydrogenase (G-6-PD) isoenzyme pattern, and HaLV *gs* antigen activity have been studied and reported for stilbestrol (DES)-induced and DES-dependent and DES-independent (autonomous) renal tumors of the Syrian hamster [1, 2]. The two autonomous tumors studied 1098 (3482) and 1098 (7528),† were obtained from the tumor bank of Dr. H. Kirkman and have been described in a review of autonomous renal tumors of the Syrian hamster by Kirkman [3].

Both dependent and autonomous tumors appear to be made up of epithelial cells, although some of the cells have a spindle-shaped appearance. The cells contain rough endoplasmic reticulum, ribosomes, mitochondria, lysosomes, lipid droplets, and are connected by desmosomes; a basal lamina is observed with some cells. The DES-dependent tumors have been reported by Li to exhibit annular nexuses not observed in normal proximal tubules [4]. The DES-dependent tumor cells demonstrate a high expression for both the slow (B) and fast (A) migrating G-6-PD isoenzyme bands. Kidney samples from areas near the primary tumors demonstrate a lower expression of the faster migrating isoenzyme (A); the autonomous tumors studied demonstrate a high expression of the slow migrating band (B) and a low or non expression of the fast migrating band (A), [1]. Non DES-treated kidney also demonstrates a low expression of isoenzyme (A) (see Results). DES-dependent tumors have a lower expression of the HaLV *gs* antigen than the DES-independent tumors.

Dodge has suggested [1, 2] that the G-6-PD isoenzyme pattern of the DES-dependent tumor might be used to indicate a unique step in tumorigenesis of the renal tumor in the hamster. The tumor while still dependent on DES, has been shown to have a low expression of HaLV *gs* activity and at this particular point in tumor progression might be vulnerable to inhibitor treatment, i.e. antiviral agents or antiestrogens.

The following paper reports the results obtained when polyriboinosinic/polyribocytidylic acid (Poly I/C) a synthetic double stranded RNA was administered i.p. and antiestrogens, ICI 46,474-*trans*-isomer of 1-(*p*-beta-dimethylaminoethoxphenyl)-1,2-diphenylbut-1-ene and U-11,100A-nafoxidine hydrochloride, were administered orally and subcutaneously, respectively. The mode of administering Poly I/C varies from species to species (i.v., i.p., s.c.) [5–7]. It has been studied by a number of laboratories [5–10], and it has been suggested that it binds to the cell membrane and promotes interferon production [9] or that it acts on cell

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†1098 (7528) was reported as 1098 (7582) in reference (2).

bound immunity [11]. The antiestrogen activity of ICI 46,474 and U-11,100A may be attributed to interference with estrogen receptor sites or possibly blockage of estrogen dependent enzymes [12, 13]. U-11,100A has been reported to compete with estradiol for the specific 4S cytosol receptor in normal and estrogenized kidney [14]. All tissues sampled were studied with the electron microscope for fine structure and by electrophoresis to determine the G-6-PD isoenzyme patterns. HaLV *gs* antigen activity was determined on a representative number of tumors.

MATERIAL AND METHODS

Material

Stilbestrol (DES)-dependent tumors were induced in golden Syrian hamsters by subpannicular implantation of 30 mg of DES for an interval of 250 or more days. Stilbestrol-induced but autonomous renal tumors (3482 and 7528) were grown in adult male hamsters for 2–3 months before becoming palpable. Poly I/C (1 mg/ml pyrogen-free saline) was obtained from Microbiological Associates, Bethesda, MD; ICI 46,474 (powder) was obtained from ICI America Inc., Wilmington, Delaware, and U-11,100A (powder) was obtained from the Upjohn Company, Kalamazoo, MI.

Methods

All tissue samples obtained were used fresh or frozen quickly and stored at −20 C for future study. Tissues were homogenized in 3 vol of glass distilled water for 1 vol of tissue in glass tissue grinders. The homogenates were spun down at 10,000 ***g*** for 10 min. Protein determination was performed on all samples. Twenty to hundred microliter aliquots were applied to acrylamide gels. Electrophoretic procedures were conducted in flat bed acrylamide systems (Ortec Inc., Oak Ridge, TN). Gels were incubated in a medium consisting of 35 mg nicotinamide adenine dinucleotide phosphate (NAPD); 25 mg nitroblue tetrazolium (NBT); 50 ml Tris/HCL buffer, pH 6.8; 600 mg glucose 6-PO_4 (dipotassium); and the reaction was activated with 12 drops of phenazine methyl sulfate. All gels were photographed for permanent records.

Electron microscopic procedures were conducted routinely as reported earlier [2].

HaLV *gs* antigen tests were conducted in the laboratory of Dr. Robert J. Huebner, Bethesda, MD, according to the procedure reported earlier [2].

Poly I/C was administered in the following way: 1 ml (1 mg/ml) was injected s.c. near the periphery of the autonomous tumors and 1 ml (1 mg/ml) was injected i.p. into hamsters carrying primary DES-induced and dependent renal tumors. Injections were given every other day. ICI 46,474 was suspended in Tween 80, 0.6 mg/ml and 0.1 ml of this suspension was mixed with apple juice and fed by medicine dropper to the hamsters daily. U-11,100A was suspended in safflower oil, 100 mg/ml, and 1 ml was injected s.c. daily, for at least 5 days. All primary, DES-dependent renal tumors were viewed prior to treatment by means of an incision in the dorsal body wall and classified as small, medium or large. After Poly I/C or antiestrogen treatment the tumor size was inspected again to note any decrease in size. Tumors were classified as small (1–5 mm), medium (1 cm), large (greater than 1 cm) and huge (greater than the size of a normal kidney). All DES-independent tumors carried subcutaneously were measured along 2 axes prior to treatment and after it.

In one experiment the primary tumors were photographed prior to Poly I/C treatment and then photographed again after Poly I/C treatment.

RESULTS

Poly I/C was used to treat a group of 12 host animals with DES-dependent renal tumors. The duration of treatment and G-6-PD isoenzyme pattern have been summarized in Table 1. The G-6-PD banding pattern of all tumors treated reverted to that observed in surrounding adjacent kidney, demonstrating a low expression of isoenzyme (A) and to that observed in kidneys from normal non DES-treated hosts which also demonstrated a very low expression or absence of the faster migrating isoenzyme (A), (Fig. 1). Tumors C-162 and C-58, deprived of DES but not given Poly I/C, maintained the isoenzyme pattern observed in DES-dependent and treated tumors. When the tumors were between 1–5 mm in size prior to Poly I/C treatment there was evidence of actual regression in size after Poly I/C treatment. An example of this regression is illustrated in Fig. 2, which shows a primary renal tumor photographed prior to Poly I/C treatment and after Poly I/C treatment. The data from 5 autonomous tumors treated with Poly I/C have been summarized in Table 2. There were no changes in isoenzyme pattern from that observed in non-Poly I/C-treated hosts since in the autonomous tumors isoenzyme (A) was observed to be absent or faintly expressed. There were also no significant decreases in macroscopic size observed.

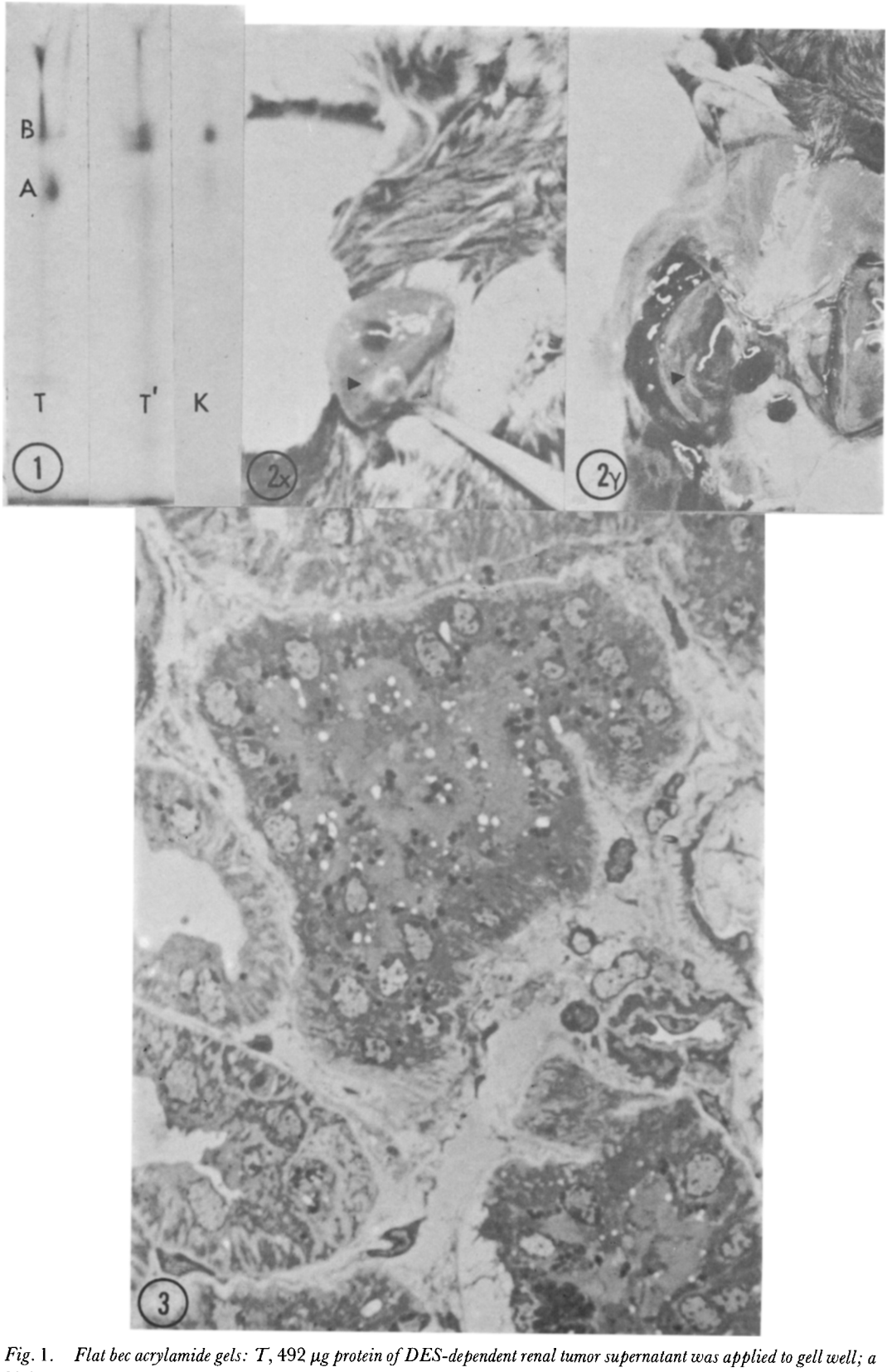


Fig. 1. *Flat bec acrylamide gels: T*, 492 μg *protein of DES-dependent renal tumor supernatant was applied to gell well; a high expression of both G-6-PD isoenzyme bands A and B is demonstrated. T'*, 1085 μg *protein of Poly I/C-treated DES-dependent renal tumor supernatant was applied to gel well; only isoenzyme B is demonstrated. K*, 722 μg *protein of non DES-treated adult male kidney supernatant was applied to gel well; isoenzyme B demonstrates a high expression and isoenzyme. A demonstrates a faint expression. The origin is at the top of photograph.*

Fig. 2. *Photographs of hamster kidney bearing DES-dependent tumor before and after, Poly I/C treatment. 2X, left kidney with medium sized tumor* (*arrow*) *prior to I/C treatment; 2Y same kidney after a total of* 12 mg *of Poly I/C was given over* 4 *weeks. The medium sized tumor indicated in the before treatment photograph has now become a cystic hemorrhagic mass* (*arrow*).

Fig. 3. *Light micrograph of* 0.500 μ *thick epon section of Poly I/C treated kidney with DES-dependent renal tumors. Tumorous proximal convoluted tubules contain dark, dense bodies and light vacuoles,* × 560.

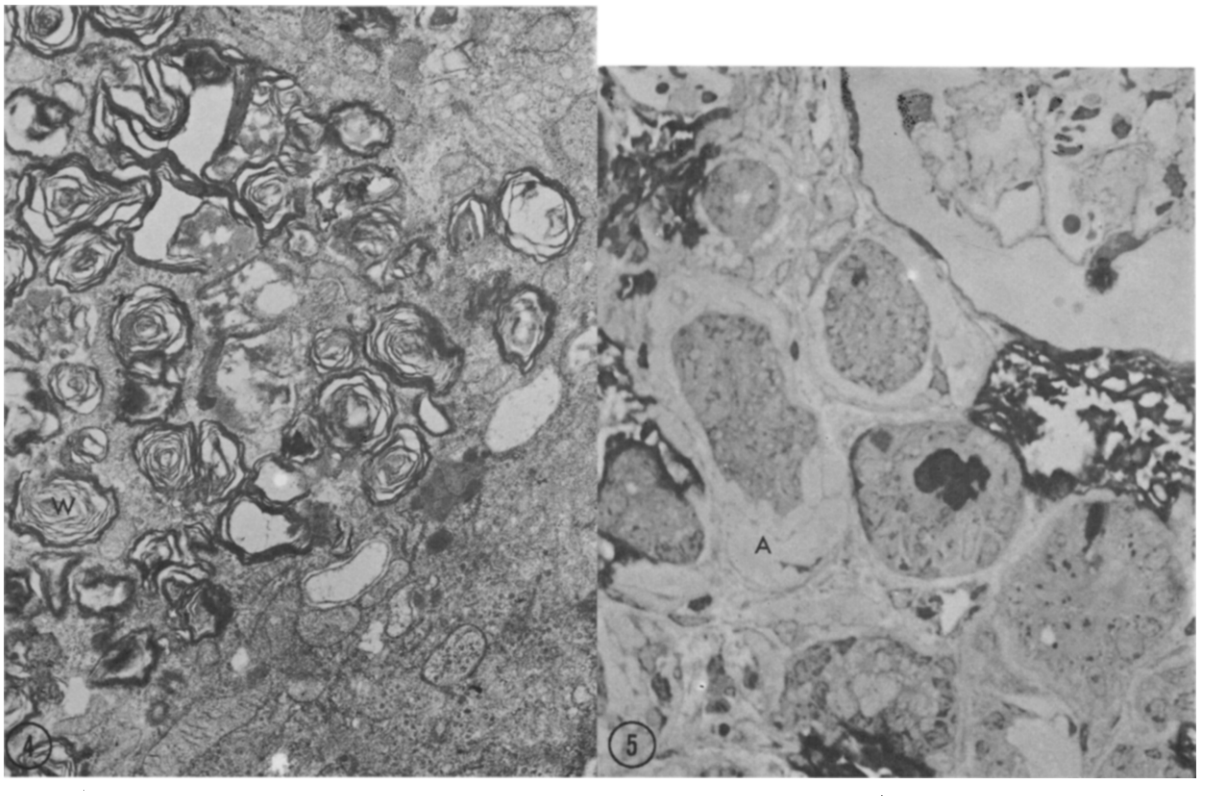


Fig. 4. *Electron micrograph of DES-dependent tumor treated with Poly I/C. The cytoplasm contains numerous membranous whorls* (*W*), × 16800.

Fig. 5. *Light micrograph of* 0.500 μ *thick epon section of Poly I/C treated kidney with DES-dependent renal tumors. A tumorous proximal convoluted tubule is surrounded by amorphous material* (*A*), × 560.

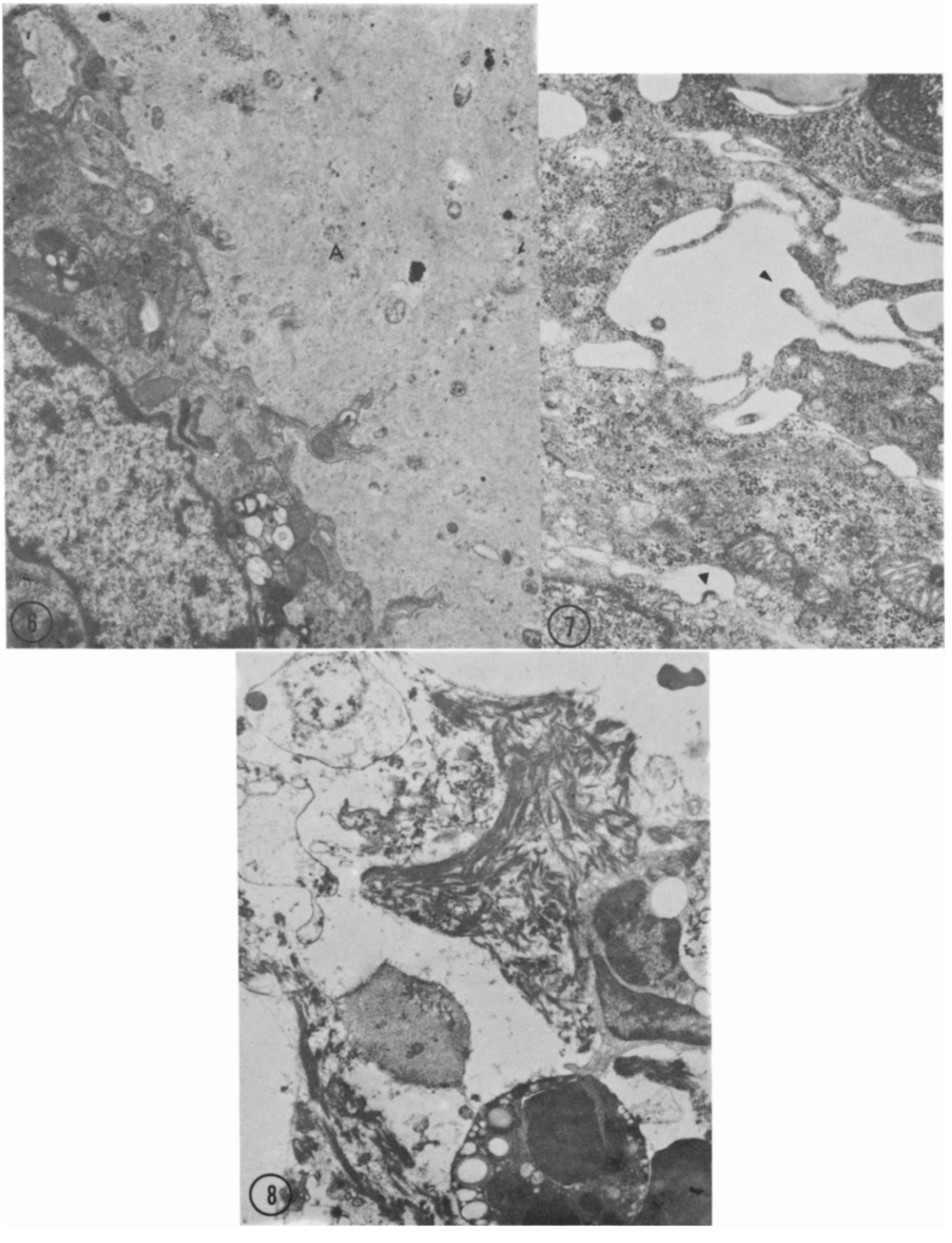


Fig. 6. *Electron micrograph of DES-dependent tumor treated with Poly I/C. The basal lamina has been replaced by amorphous material* (*A*), × 16,800.

Fig. 7. *Electron micrograph of healthy area of DES-independent renal tumor treated with Poly I/C. Arrow indicate virus-like C type RNA particles budding from cell surfaces*, × 24,000.

Fig. 8. *Electron micrograph of necrotic area of DES-independent renal tumor treated subcutaneously with Poly I/C*, × 3920.

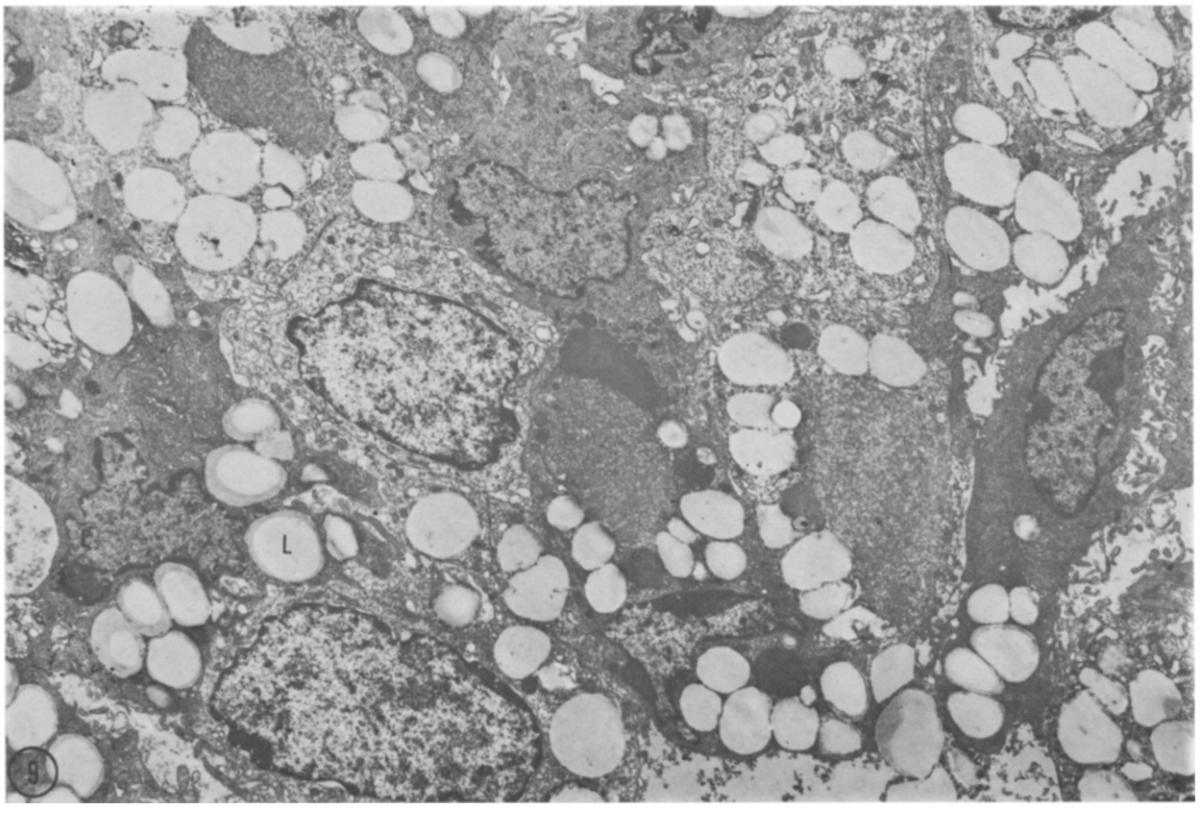
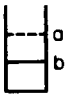
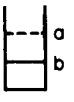
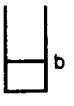
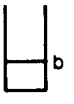
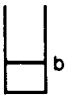
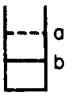
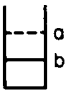
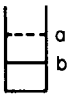
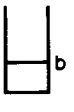
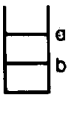
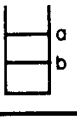
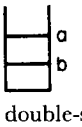


Fig. 9. *Electron micrograph of DES-dependent renal tumor treated with U*-11-, 100*A. The cytoplasm of the cells is filled with varying number of lipid droplets*, × 7280.

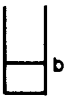
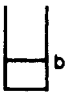
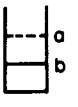
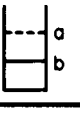
Table 1. *Primary renal tumors DES-dependent*

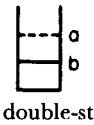
Animal code	Inhibitor treatment	G-6-PD isoenzyme pattern*
c-126	†Poly I/C i.p. 55 mg/10 days	
c-128	5 mg/10 days	
c-124	‡5 mg/10 days	
c-129	‡5 mg/10 days	
c-133	5 mg/10 days	
c-130	30 mg/60 days	
c-100	14 mg/48 days	
c-102	24 mg/48 days	
c-6	10 mg/10 days	
c-14	‡17 mg/34 days	no data
c-161	‡23 mg/46 days	no data
c-167	12 mg/24 days	no data
c-162	§DES removed 20 days	
c-58	§DES removed 50 days	

*DES-dependent renal tumor G-6-PD isoenzyme pattern 

†Poly I/C = polyriboinosinic/polyribocytidylic acid (synthetic double-stranded RNA), (Microbiological associates); 1 mg was injected 3 × each 7 days.
‡DES removed also.
§Primary DES-removed controls.

Table 2. Autonomous DES-independent tumors

Animal code	Inhibitor treatment	G-6-PD isoenzyme pattern*
	†Poly I/C injected into periphery of tumor	
c-80	5 mg/10 days	no data
c-50	12 mg/24 days	
c-051	6 mg/12 days	
c-136	15 mg/30 days	
c-132	16 mg/32 days	

*DES-independent renal tumor G-6-PD isoenzyme pattern. 

†Poly I/C = polyriboinosinic/polyribocytidylic acid (synthetic double-stranded RNA). (Microbiological associates): 1 mg was injected 3 × each 7 days.

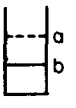
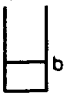
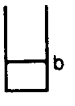
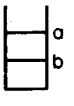
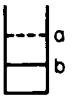
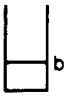
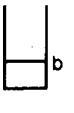
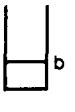
The antiestrogen agents were used to treat 9 host animals with DES-dependent renal tumors (Table 3). All but one of the tumors showed a return of the G-6-PD isoenzyme pattern to that observed in adjacent kidney. One animal showed a regression in the size of the tumor.

Both Poly I/C and antiestrogen-treated renal tumors were tested for HaLV *gs* antigen activity by Dr. Robert J. Huebner, Bethesda, MD (Table 4). Four Poly I/C-treated DES-dependent tumors had complement fixation titers (CFT) of 2 or less than 2, compared to a CFT of 4–8 reported for DES-dependent renal tumors in an earlier paper [2]; therefore Poly I/C treatment appeared to cause some decrease in the CFT of DES-dependent tumors. The CFT of a tumor no longer supported by DES treatment and not treated with Poly I/C did not show a decrease in the titer. Three Poly I/C treated DES-independent autonomous tumors had CFTs from 8–16, which were within the range reported for non-Poly I/C-treated tumors; therefore Poly I/C did not affect the CFT of these tumors.

Cytological changes were observed with light and electron microscope studies of DES-dependent tumors treated with Poly I/C. In a light micrograph of the tumor, vacuoles and densities were observed in some of the tumor cells (Fig. 3). At the fine structural level cells were observed to contain membranous whorls often filling up most of the cytoplasm (Fig. 4). Non-Poly I/C treated DES-dependent tumors processed for microscopy in the same manner have never been observed to contain these whorls. Amorphous material was observed in some areas of the tumor where basal lamina was still apparent (Fig. 5); it occurred either between the basal lamina and cell membrane or outside the basal lamina. But where the basal lamina was no longer apparent the amorphous material seemed to have replaced it (Fig. 6). Poly I/C treatment did not appear to alter the cytological nature of the adjacent kidney tissue.

DES-independent renal tumors treated with Poly I/C had regions of unchanged tumor tissue (Fig. 7) and regions where there was a great deal of necrosis (Fig. 8). Focal irritation due to injections of Poly I/C every other day may have accounted for the necrosis; or the focal changes

Table 3. Primary renal tumors DES-dependent

Animal code	Inhibitor treatment	G-6-PD isoenzyme pattern
	Antiestrogen *ICI 0.05 mg administered orally each day	treatment
c-106	Citrate salt 0.4 mg/8 days	
c-158	†Citrate salt 1 mg/20 days	
c-159	†Citrate salt 3 mg/60 days	
c-150	†Free base 1 mg/20 days	
c-10	†Free base 2/25 mg/45 days	no data
	‡U-11, 100A injected subcutaneously 1 mg each day	
c-116	5 mg/5 days	
c-160	†20 mg/20 days	
c-148	†30 mg/30 days	
c-41	15 mg/15 days	

*ICI 46,474 = *trans*-isomer of 1-(*p*-beta-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene (ICI America Inc.).
†DES removed also.
‡U-11, 100A-nafoxidine hydrochloride (The Upjohn Company).

Table 4. Complement fixation test

Tumor	Inhibitor treatment	Complement fixation titer (reciprocal) with 4 units indicated serum
Primary renal tumors DES-dependent		
1	*Poly I/C i.p. 24 mg/48 days	2
2	*Poly I/C i.p. 24 mg/48 days	2
3	*Poly I/C i.p. 17 mg/34 days	<2
4	*Poly I/C i.p. 30 mg/60 days	2
5	Primary with DES Rx stopped for 50 days	4
6	†U-11, 100A 15 mg/15 days	16
7	‡ICI 46,474 1 mg/20 days	8
8 Adjacent kidney	*Poly I/C i.p. 30 mg/60 days	<2
DES-independent renal tumors	*Poly I/C injected into periphery of transplant	
1098 (3482)	15 mg/30 days	16
1098 (3482)	16 mg/32 days	16
1098 (7582)	6 mg/12 days	8

*Poly I/C = polyriboinosinic/polyribocytidylic acid (synthetic double-stranded RNA), (Microbiological Associates); 1 mg was injected 3 × each 7 days.
†U-11, 100A = nafoxidine hydrochloride, (The Upjohn Company).
‡ICI 46,474 = *trans*-isomer of 1-(*p*-beta-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene (ICI America Inc.).

may have resulted from vascular changes brought about by the Poly I/C treatment as suggested in ref. [4].

DES-dependent tumors treated with antiestrogens demonstrated cytoplasmic vacuoles which often contained lipid (Fig. 9). When the vacuoles filled most of the cytoplasm, membranous whorls were sometimes present.

DISCUSSION

In the progression of initially hormone-dependent tumors to hormone-independent tumors it would be clinically useful to be able to arrest tumorigenesis during periods of hormone vulnerability. It has been suggested earlier [1] that the isoenzyme pattern of G-6-PD of DES-induced and dependent renal tumors could be used as a marker of vulnerability. The data reported in this paper support this hypothesis in that both Poly I/C and antiestrogen treatment caused a reversal in G-6-PD isoenzyme pattern in DES-induced and dependent renal tumors to that of surrounding non-tumorous adjacent kidney tissue and non DES-treated kidney. Li *et al.* [15] reported 4 G-6-PD isoenzyme bands for the DES-induced and dependent renal tumors. The slower migrating bands described in his work correspond to the slower migrating diffuse staining zones observed in both DES-dependent tumors and Poly I/C treated DES-dependent tumors (Fig. 1). However it is the fastest migrating band (A) which is reduced in expression after Poly I/C treatment. Schmulker [16] reported that, when ammonium persulfate was used in the polyacrylamide gel system, the expression of the slower migrating positive region was enhanced in rat tissue. However, since ammonium persulfate was used routinely in both the work reported by Dodge (1973) and Li (1975) this could not explain the difference found by Dodge and Li.

The decrease of the HaLV *gs* CFT of Poly I/C-treated DES-induced and dependent renal tumors though small occurred concomitantly with the isoenzyme change. Therefore, during the period of vulnerability delimited by the G-6-PD isoenzyme pattern reversal there existed also an interval when HaLV antigen production could be influenced by the secondary action of Poly I/C. The period of vulnerability did not appear to extend to the DES-independent state since in the autonomous renal tumors the G-6-PD isoenzyme pattern and HaLV activity were not affected by Poly I/C treatment.

Cytological changes in the DES-dependent renal tumors, and in some cases actual tumor regression, were also observed in addition to the isoenzyme change and HaLV CFT reduction. Since Poly I/C-treated primary DES-dependent tumors demonstrated abundant extracellular

amorphous material and the primary DES-dependent tumors did not, it would be of interest to determine in future studies if the type of collagen produced by the DES-dependent tumor changed after Poly I/C treatment. Indeed increased production or accumulation of extracellular material in the Poly I/C-treated tumors might be responsible for the reported tumor regression. Therefore, in this preliminary study of the effects of Poly I/C and antiestrogens on DES-induced renal tumors the G-6-PD isoenzyme banding pattern appeared to be useful as a marker isoenzyme to characterize a stage in tumorigenesis vulnerable to inhibitor treatment.

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Preliminary Results of a Randomized E.O.R.T.C. Study* Comparing Radiotherapy and Concomitant Bleomycin to Radiotherapy Alone in Epidermoid Carcinomas of the Oropharynx

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Abstract—*The purpose of this randomized trial was to verify some experimental and clinical data suggesting a possible potentiating effect of bleomycin when used concomitantly with radiotherapy. Out of* 220 *patients with a biopsy proven epidermoid carcinoma of the oropharynx*, 186 *evaluable cases are reported here. One group of* 87 *patients was treated with radiotherapy alone (Cobalt* 60 ~ 6400 rad ~ 7–8.5 *weeks), the other group of* 99 *patients received radiotherapy combined with bleomycin, the latter administered at the dose of* 15 mg *i.m. twice a week for* 5 *weeks (total dose:* 150 mg).

Analysis of the treatment groups showed their comparability with regard to the most important prognostic factors (TNM, sites of primary, sex, age...). Complication rates of mucositis and epidermitis were significantly increased (71%) *in the radiotherapy + bleomycin group and were considered responsible of frequent denutrition and weight loss in this group. Such side effects necessitated a delay of radiotherapy in* 22% *of patients and definitive interruption in* 5% *in the combined treatment group whereas in the radiotherapy alone group no interruption of treatment was recorded and only* 5 *treatments* (6%) *were postponed.*

Considering tumor regressions measured 6 *weeks after completion of radiation therapy, total regression rates were not significantly different in both groups as far as primary tumor* (67.9–67%) *or neck nodes* (49–62%) *are concerned. Survival curves obtained by the actuarial method showed the same* 50% *survival in both groups at* 15 *months of follow-up.*

INTRODUCTION

RADIOTHERAPY is the usual treatment of epidermoid carcinomas of the oropharynx. Although the introduction of high energy irradiation through the use of cobalt 60 and electron therapy has allowed a better control of these tumors, their long term prognosis remains poor with a survival rate not exceeding 30% at 5 yr. The prognosis is even worse for infiltrative carcinomas of the base of the tongue and in cases with neck node involvement [1].

During the past 10 yr interest has grown in the use of bleomycin which accumulates in the epithelial layers and has been shown to be effective on carcinomas of the head and neck [2].

The sensitivity of tumor cells to the combined action of bleomycin and irradiation has been investigated in experimental studies [3]. In several models the cumulative effect of bleomycin and irradiation has been observed [4]. Other studies investigating a possible synergic action of bleomycin provided results which depended on the type of cellular strain used [5]

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and on the mode of combination of bleomycin with irradiation, either simultaneously or sequentially [6].

Pilot clinical studies recently carried out on patients with carcinomas of the head and neck suggest a cumulative antitumor effect when bleomycin is given in conjunction with radiotherapy [7].

The aim of the present randomized clinical trial is to compare bleomycin administered during irradiation to radiotherapy alone with regard to the rate of remission and duration of survival in carcinomas of the oropharynx.

MATERIAL AND METHODS

The following criteria were established for the entry of patients on study:

1. All patients must have had a biopsy proving advanced squamous cell carcinoma of the oropharynx.
2. The following localizations were included: the base of the tongue, the tonsillar fossa, the posterior wall and the soft palate.
3. The largest diameter of the primary tumor must have measured at least 2 cm (T2 of the UICC classification) or the tumor must have had an infiltrative character irrespective of its size.
4. Patients with neck node involvement ($N1$, $N2$, $N3$) were included in the trial, however those with distant metastases ($M1$) or a second primary lesion (other than skin carcinomas) were excluded.

Patients were randomized to receive one of two treatments:

1. Telecobalt alone: the schedule for the primary tumor and metastatic lymph nodes was a maximum total dose of 7000 rad in 7–8.5 weeks while regional uninvolved nodes were to receive 5000–5500 rad in 5–6 weeks.
2. Radiotherapy as above plus concomitant bleomycin (BLM): 15 mg of BLM to be administered by i.m. or i.v. shots twice a week from the start of radiotherapy for a period of 5 weeks (total dose 150 mg in 5 weeks). Each injection of BLM was to be given 2 hr prior to the start of each session of radiotherapy and prophylactic polaramine injections were to be given to prevent allergic reactions. The degree of tumor response (100%, $\geq 50\%$, $< 50\%$, none or growth) was to be evaluated 6 weeks after completion of radiotherapy by comparing the residual volume of the primary tumor and the invaded nodes to the initial volume. The second endpoint of the study is the comparison of the survival rates in the two treatment groups.

The Statistical Unit of the Institut Gustave-Roussy in Villejuif was responsible for the handling of the data. Statistical analysis was carried out mainly in Villejuif and partly at the E.O.R.T.C. Data Center in Brussels. Tables of random numbers were used to prepare randomization envelopes for each institution. The randomization was stratified according to institution and balanced after every 4 patients within each institution. To date all patients have reached at least 1 yr of follow-up and actuarial survival curves have been calculated for each treatment group.

RESULTS

1. *Distribution of patients entered into this study*

Two hundred and twenty-four patients entered into this study from 1 April, 1973 to 31 December, 1974. Thirty-eight patients were excluded from this analysis, 31 (14%) due to protocol violations and 7 (3%) due to incomplete data. Thus 186 patients (83%) were evaluable, 87 in the group receiving radiotherapy alone and 99 in the group receiving bleomycin and radiotherapy. Table 1 gives the distribution of evaluable patients by institution and treatment.

The 2 treatment groups were comparable with regard to the principal prognostic factors. With regard to age and sex, the average age is 57 yr in both groups while females comprise 6% of the radiotherapy group and 13% of the group receiving radiotherapy and bleomycin. With regard to the distribution of patients according to the initial tumor site, Table 2 shows the groups to be comparable with carcinomas of the tonsillar fossa (44%) and the base of the tongue (36%) being the most frequent. The distribution of patients according to the tumor extent, 1 site or more than 1 site, is given in Table 3 and it is seen that tumors are limited to 1 site in 32% of the patients. Tumors with deep invasion (infiltrative character) represent 78% of the patients in the bleomycin group and 86% of the patients in the radiotherapy alone group. The distribution of patients according to the UICC clinical classification of neck nodes is given in Table 4 and it is likewise seen that the treatment groups are comparable with respect to the N classification.

2. *Treatment received*

(a) *Radiotherapy.* The primary tumor was to receive a maximum total dose of 7000 rad in a

Table 1. *Distribution of evaluable patients by institution and treatment*

Institutions (Identification No.)	Total	Treatment groups	
		XRT	BLM + XRT
Institut Jules Bordet. Bruxelles (1)	5	1	4
Institut Gustave-Roussy, Villejuif (2)	35	17	18
Centre Léon-Bérard, Lyon (3)	54	25	29
Centre Anticancéreux, Marseille (4)	4	2	2
Hôpital Régional, Lille (5)	17	8	9
Hôpital St André, Bordeaux (7)	11	6	5
Centre Antoine-Lacassagne, Nice (8)	10	4	6
Hôpital Tenon, Paris (9)	9	4	5
A. van Leeuwenhoek Ziekenhuis, Amsterdam (10)	6	4	2
Krankenhaus Neukölln, Berlin (12)	6	4	2
Istituto di Oncologia, Torino (14)	2	1	1
St Radboud Ziekenhuis, Nijmegen (15)	2	—	2
Centre Oscar-Lambret, Lille (16)	25	11	14
	186	87	99

Table 2. *Distribution of patients according to the initial tumor site*

Tumor site	Treatment groups		Total (%)
	XRT (%)	BLM + XRT (%)	
Tonsillar fossa	40 (46.0)	42 (42.4)	82 (44.1)
Anterior pilar	—	1 (1.1)	1 (0.5)
Posterior pilar	3 (3.5)	5 (5.1)	8 (4.3)
Base of tongue	29 (33.3)	38 (38.4)	67 (36.0)
Amygdaloglossal sulcus	7 (8.1)	3 (3.0)	10 (5.4)
Soft palate	2 (2.3)	8 (8.0)	10 (5.4)
Vallecula	3 (3.4)	1 (1.0)	4 (2.2)
Oropharynx—not specified	3 (3.4)	1 (1.0)	4 (2.2)
	87 (46.8)	99 (53.2)	186

Differences are not significant.

Table 3. *Distribution of patients according to tumor extent*

Treatment groups	Tumor extent		Total
	1 site	More than 1 site	
XRT	24 (28%)	63 (73%)	87
BLM + XRT	35 (35%)	64 (64%)	99
Total	59 (32%)	127 (68%)	186

Differences are not significant.

Table 4. *Distribution of patients according to the UICC clinical classification of neck nodes*

Treatment groups	N_0	$N_{A(1,2)}$	$N_{B(1,2)}$	N_3	Total
XRT	24 (27.6%)	7 (8%)	28 (32%)	28 (32%)	87
BLM+XRT	23 (23%)	16 (16%)	27 (27%)	33 (33%)	99
	47 (25%)	23 (12%)	55 (30%)	61 (33%)	186

Differences are not significant.

period of 7–8.5 weeks. The actual distribution of the total dose received in each treatment group is given in Fig. 1. The average total nominal dose received was 6403 ± 204 rad in the group receiving radiotherapy alone and 6288 ± 168 rad in the group receiving bleomycin (difference not significant). In 30% of the patients receiving radiotherapy alone and in 25% of the patients receiving bleomycin, the total duration of radiotherapy (Fig. 2) was shorter than the scheduled 7 week period. In fact, the average duration of radiotherapy was 47 ± 2 days in the radiotherapy group and 52 ± 2 days in the bleomycin group [difference significant at $P = 0.01$ (2 sided test)]. This difference can be attributed to the more frequent delays in radiotherapy due to side effects experienced by the bleomycin group (Table 5). Treatment was not prematurely stopped in any of the patients in the radiotherapy alone group while treatment was stopped early in 5 patients in the bleomycin group due to severe mucositis and epidermitis within the field of irradiation. Such side effects were also responsible for postponement of radiotherapy courses in 5 patients receiving radiotherapy alone and in 25 patients receiving radiotherapy and bleomycin ($P < 0.001$). The nominal single dose (NSD) was calculated for patients selected from three different Institutions (Lille, Lyon and Villejuif). The average NSD varied considerably from one institution to another and was in most cases lower than the predetermined NSD for patients in the bleomycin group (Fig. 3). This figure also shows that Lyon systematically restricted the total amount of radiotherapy in the bleomycin group in order to obtain a uniform duration of treatment in both groups. In Lille, where the total dose of radiotherapy was considered as the main criteria of treatment, radiotherapy courses were delayed and extended over longer periods of time in the bleomycin group. In Villejuif, the NSD received was very close to the predetermined NSD in both treatment groups.

(b) *Bleomycin.* Bleomycin treatment began with the first course of radiotherapy in 97% of the patients, while, in 3%, the first dose of bleomycin was not given until after the first week of radiotherapy. Sixty-eight per cent of the patients received the prescribed dose of 150 mg while in 12% a maximum of 90 mg was administered due to local reactions (Fig. 4).

3. *Complications due to treatment* (*Table* 6)

The overall rate of secondary reactions was 23% in the group radiotherapy alone and 78% in the group radiotherapy and bleomycin ($P < 0.01$). This difference is due, in part, to the

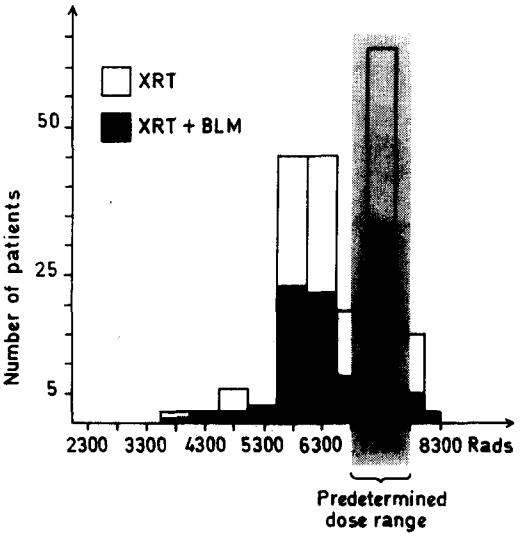

Fig. 1. *Distribution of total dose of radiotherapy received.*

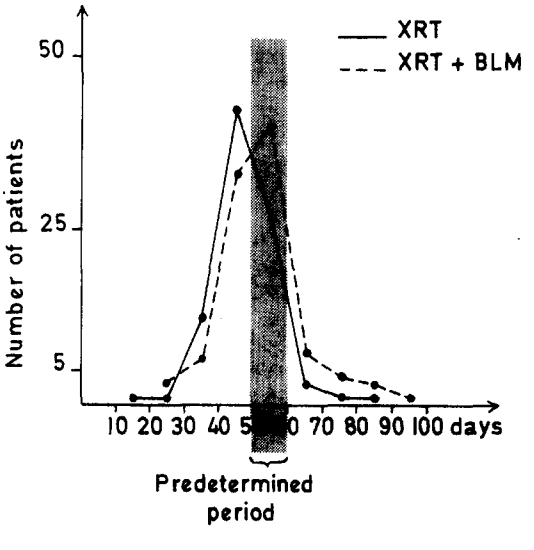

Fig. 2. *Total duration of radiotherapy.*

Table 5. *Delay in radiotherapy due to side effects*

Treatment groups	XRT postponed (%)	XRT stopped prior to completion (%)
XRT	5 (6)	0 (0)
BLM + XRT	25 (22)	5 (5)

Difference significant at $P < 0.01$ and $P = 0.095$.

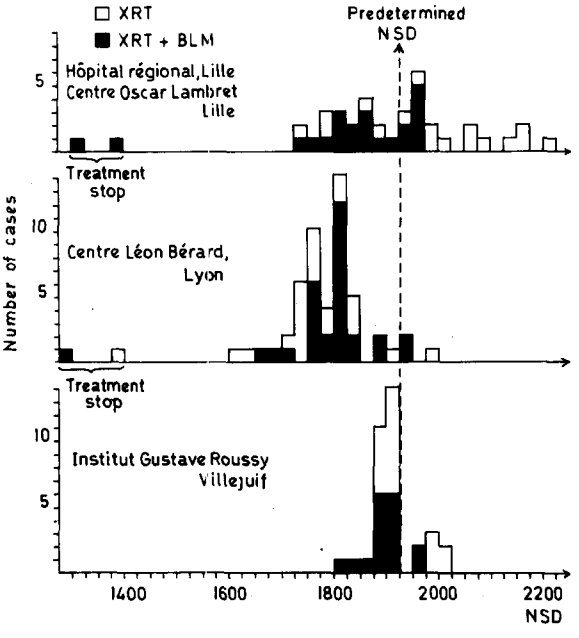

Fig. 3. *Distribution of NSD in three institutions.*

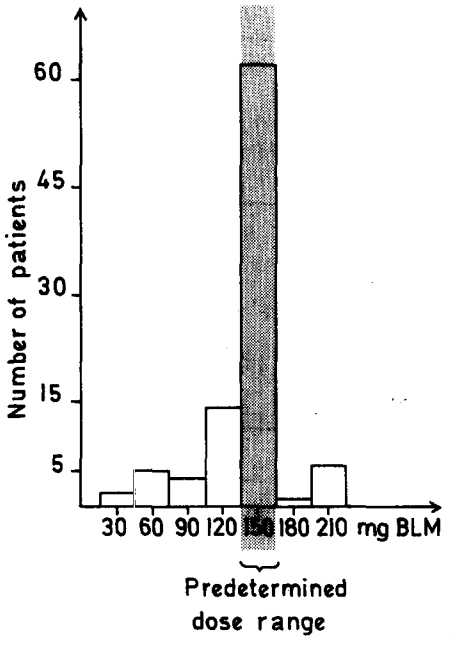

Fig. 4. *Distribution of total BLM dose.*

difference in the local toxic effects such as mucositis and mucositis with epidermitis experienced in the 2 groups. Twenty-one per cent of the patients in the radiotherapy alone group and 72% of the patients treated with bleomycin experienced mucositis and/or epidermitis ($P < 0.01$). The low incidence of local reactions in the radiotherapy alone group may be explained by the fact that these restrictions may have been minimized by the clinician and biased by the design of the forms.

Although slightly more common in the bleomycin group, there was no significant difference between the groups with respect to skin rash, pneumopathy, hemorrhage, severe dysphagia or fever. Weight loss (18% vs 6%) and profound weakness (19% vs 1%) are significantly more common in the group receiving bleomycin, where normal nutrition was impaired due to mucositis and in several patients necessitated feeding by a nasogastric tube.

4. *Tumor response*

The time of evaluation of the tumor response varied from less than 30 days to more than 3 months after completion of radiotherapy (Table 7). The average time of tumor evaluation was approximately 60 days: however, 13% were evaluated after 90 days. There is, however, no significant difference between the groups with respect to the time of evaluation. The response rate of the primary tumor is given in Table 8. A 100% response rate was observed in approximately 67% of the patients in both groups and there is no significant difference between the treatments with respect to the degree of response. There are also no significant differences between the response rates to the treatments when the tumor site, the tumor extent and the dose of bleomycin received were taken into consideration. There was likewise no significant difference between the treatments with respect to the response rate of clinically involved lymph nodes (Table 9).

Actuarial survival curves are given in Fig. 5 and indicate that there are currently no significant differences between treatment groups with respect to the duration of survival. The median duration of survival is approximately 15 months in each group.

DISCUSSION

This study, the first trial undertaken by the E.O.R.T.C. Head and Neck Cooperative Group shows the interest of people working together in a trial conducted on an international scale. Thirteen Institutions from 5 different countries entered 224 patients in less than 2 yr. Thirty-one patients (14%) have been definitively excluded from the trial due to protocol violations while 7 (3%) have been excluded from these results due to incomplete data.

The protocol was followed without deviation in 43% of the patients in the radiotherapy alone group and in 22% of the patients receiving bleomycin. The total dose and duration of radiotherapy as specified in the protocol was not generally attained in patients in either of the 2 treatment groups. Several Centers were reluctant to modify their own schedule of fraction-

Table 6. Complications due to treatment

Complications	BLM + XRT (%)	XRT (%)	Statistical difference S or N.S.
—Mucositis	47 (47.4)	11 (12.6)	S ($P<0.001$)
—Epidermitis	2 (2.0)	3 (3.4)	N.S.
—Mucositis with epidermitis	22 (22.2)	4 (4.6)	S ($P<0.01$)
Total	71 (71.7)	18 (20.7)	S ($P<0.01$)
—Skin rash	4 (4.0)	1 (1.1)	N.S.
—Pneumopathy	2 (2.0)	1 (1.1)	N.S.
—Hemorrhage	3 (3.0)	0 (0)	N.S.
—Severe dysphagia	1 (1.0)	0 (0)	N.S.
—Fever	2 (2.0)	0 (0)	N.S.
—Profound weakness	19 (19.1)	1 (1.1)	S ($P<0.01$)
—Weight loss	18 (18.1)	5 (5.7)	S ($P<0.05$)
Total	77 (78.0)	20 (23.0)	S ($P<0.001$)

Table 7. Delay between end of XRT and evaluation of tumor response

Treatment group	< 1 month (%)	1 to 2 months (%)	2 to 3 months (%)	> 3 months (%)
XRT	2 (2.4)	32 (38.0)	41 (49.0)	9 (10.7)
BLM + XRT	1 (1.0)	39 (41.5)	38 (40.0)	16 (17.0)

Difference not significant.

Table 8. Tumor response rate (regression rate)

Treatment groups	Primary Tumor			
	100 (%)	≧50 (%)	<50 (%)	None or growth (%)
XRT	57 (67.9)	11 (13.1)	12 (14.3)	4 (4.7)
BLM + RXT	63 (67.0)	16 (17.0)	13 (13.8)	4 (2.2)

Difference not significant.

Table 9. Lymph node response rate (regression rate)

Treatment groups	Involved neck nodes			
	100 (%)	≧50 (%)	<50 (%)	None or growth (%)
XRT	28 (49)	15 (26)	7 (12)	7 (12)
BLM + XRT	41 (62)	9 (14)	11 (17)	5 (7)

Difference not significant.

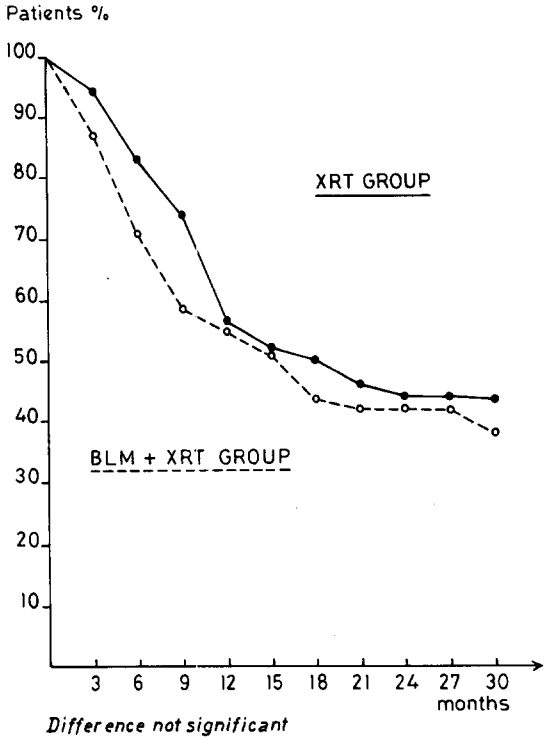

Fig. 5. Survival curves by the actuarial method.

ation while other centers delayed the continuation of radiotherapy in the presence of side effects in order to minimize local reactions. The variations from the protocol are, however, of the same frequency in the 2 treatment groups.

The concomitant use of bleomycin with radiotherapy as specified in the protocol was in fact not well tolerated and does not appear to be realizable. The incidence of both local and general complications was significantly higher in the bleomycin group, even for doses of less than 90 mg. In this group, radiotherapy was often delayed during the third week and had to be stopped prematurely in 5 patients. Modifications of radiotherapy received, occurred on a much lower scale in the radiotherapy alone group.

Analyzing the therapeutic results from a pragmatic point of view, that is considering the trial as it was actually carried out, bleomycin does not appear to enhance the efficacy of radiotherapy on advanced carcinomas of the oropharynx with respect to either the primary tumor or the invaded lymph nodes. With respect to the duration of survival there is no significant difference between the groups. However, initially it seems that patients with a poor prognosis die quicker in the group receiving bleomycin. This might be explained by the side effects of radiotherapy (dysphagia by mucositis) which are further aggravated through the concomitant administration of bleomycin. There is thus a higher initial risk of death in the bleomycin group brought about by the increase of major complications. It remains to be seen whether or not the long term survival of patients in the 2 groups will be the same now that 1 yr of follow-up has been completed on all patients.

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Serological Characterization of a Putative Human C-Type Oncornavirus by Means of the Sepharose Bead Immunofluorescence Assay*

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Abstract—*A putative human C-type oncornavirus isolate was found to be closely related to the simian sarcoma virus by means of the Sepharose bead immunofluorescence assay.*

Sepharose-beads coupled with antisera directed against purified major internal proteins of C-type oncornaviruses were incubated first with tissue culture supernatants and thereafter with antisera conjugated with fluorescein isothiocyanate. The fluorescence of individual beads was then measured microfluorometrically. This technique is a highly sensitive and reproducible assay for the detection of viral proteins.

The SKA21-3 culture of rabbit cornea cells, transformed by a murine sarcoma virus pseudotype with a presumably human derived C-type oncornavirus as helper proved to produce abundant amounts of proteins related to the p28 of simian sarcoma virus but not to the p30 of murine leukemia virus. When cells from a tumorous lymph node induced in a rat by the SKA21-3 virus were cocultivated with a canine thymus cell line, A7573, high concentrations of viral proteins were detected in the supernatant after a few passages. The same result was obtained when a leukemic lymph node from a rat inoculated with the presumed human virus, free of the murine sarcoma virus, was cocultivated with the human A204 line.

INTRODUCTION

C-TYPE oncornaviruses have been isolated from human leukemic bone marrow cells by cocultivation with animal cells in our laboratory [1, 2]. These isolates can serve as helper viruses for replication-defective murine sarcoma virus (MSV). They may therefore rescue the MSV genome from nonproducing transformed cells. The resulting MSV pseudotype is able to transform cells which are permissive to the human-derived helper virus. The focus forming activity of this new MSV pseudotype can be neutralized by a goat antiserum against the simian sarcoma virus, indicating that the human helper virus has envelope antigenic determinants in common with the simian sarcoma virus (SiSV) [2].

A clone of rabbit cornea (SIRC) cells transformed by such a derived MSV pseudotype, designated as SKA21-3, became a high producer of C-type oncornavirus (see Fig. 1 for the origin of this line). This made the further biological and serological characterization of the virus possible. The MSV-pseudotype produces angiomatous, fibrohistiocytic sarcomas in rats 2 months after inoculation. These lesions are typical for the Kirsten strain of MSV used in this investigation.

The defective MSV genome can be eliminated from the SKA21-3 virus by endpoint dilution. Human A204 cells infected with a high dilution of this virus produced C-type particles, which had no focus-forming activity in those cell lines which were readily transformed by SKA21-3 virus. Virus isolated from the A204 cell line (A204SKA) induces lymphosarcomas with leukemic involvement of the peripheral blood in rats in the absence of typical MSV lesions [3].

The present paper concerns further serological characterization and detection of this human-derived virus using a new immunological technique: the Sepharose bead immunofluorescence assay (SBIA) [4, 5].

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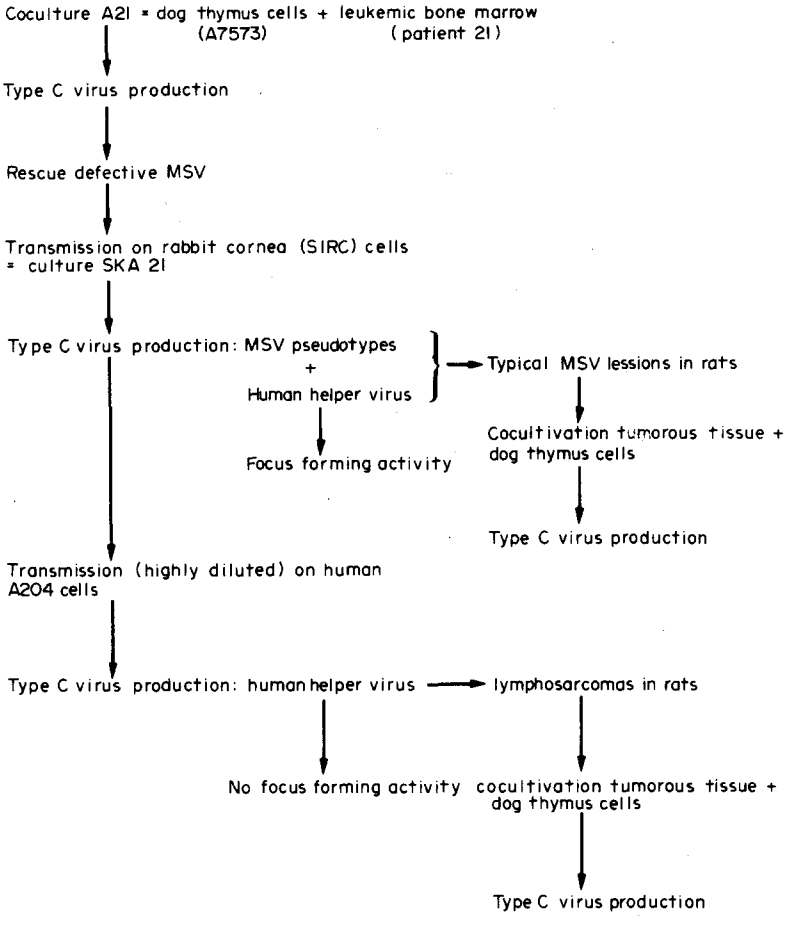

Fig. 1. Scheme of isolation procedure and subsequent passages of a human tissue-derived C-type oncornavirus.

MATERIAL AND METHODS

Cell cultures

The cell lines SKA21-3 and A204(SKA) were grown in plastic 75 cm^2 flasks (Costar 3075, Cambridge, Mass.) in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum (Flow, Irvine, Scotland). Rat embryonic fibroblasts infected with SiSV were grown under the same conditions in a separate laboratory building of the Primate Center TNO, Rijswijk, The Netherlands. SiSV-infected NC37 cells were grown in suspension in Dulbecco's medium supplemented with 15% fetal calf serum. Manipulation of all of these cultures was done under strict biohazard conditions. Murine BALB/3T3 cells infected with Rauscher murine leukemia virus (RLV) were grown in the main tumor virological laboratory building. Uninfected SIRC, BALB/3T3, canine A7573, human A204 cells and WAG/Rij rat embryonic fibroblasts were cultured in a special tissue culture unit.

Cocultures of tumor cells with uninfected cell lines were prepared in a separate laminar flow cabinet. The cells were seeded in equal ratio (2×10^5/ml).

Viruses and antisera

Simian sarcoma virus and Rauscher murine leukemia virus, purified by density gradient ultracentrifugation and goat antisera directed against the major internal protein (p30) of each virus were kindly provided by Dr. J. Gruber of the Office of Program Resources and Logistics, Viral Oncology, National Cancer Institute, Bethesda, Maryland, U.S.A.

Focus formation in vitro

Supernatants of cultures were clarified by centrifugation for 15 min at 10,000 ***g*** and then filtered through a millipore filter (0.45 μm pore size). Cultures for focus formation, inoculated at 2×10^5 cells per 60 mm Petri dish 24 hr prior to infection, were treated with DEAE-dextran (25 μg/ml) for 30 min. The cultures were then incubated with two-fold dilutions of tissue culture fluids. Dilutions were made in fresh medium containing polybrene (2 μg/ml). Eight days after infection, foci of transformed cells

were counted unstained with the aid of an inverted microscope (75 ×).

Coupling of antisera to Sepharose beads

Before coupling, the antisera were subjected to chromatography on QAE-Sephadex A50 (Pharmacia, Uppsala, Sweden) in ethylenediamine–acetate (pH: 8.0; *I*: 0.1) [6]. The isolated IgG fractions were dialyzed against phosphate buffered saline (PBS). The IgG was then coupled covalently to Sepharose 4B beads (Pharmacia) by the cyanogen bromide method of March *et al.* [7], using 2–3 mg protein per 1 ml of activated beads. Residual active groups were deactivated by treatment of the beads for 6 hr with 0.5 M ethanolamine in 2% sodium hydrogen carbonate, pH 9.5. After coupling and deactivation, the beads were washed and stored in PBS with 0.01% merthiolate at 4°C.

Sepharose bead immunofluorescence assay of viral antigens

The method as developed for the quantitative assay of immunoglobulins [4, 5] has been adapted to the detection of viral antigens. Fifty microlitres of a 1% suspension of beads (1 ml bead pack to 100 ml of PBS) coupled with IgG were incubated for 1 hr with 50 μl of a cell-free sample, diluted in PBS containing 2% bovine hemoglobin in flat bottom microtitration plates under continuous agitation. After repeated washing, the beads were incubated with one or the other of the antisera conjugated with fluorescein isothiocyanate (FITC).

After repeated washing, the individual bead fluorescence was measured with a microfluorometer. At least 5 beads were measured per dilution. A calibration curve was prepared by incubation of the beads with different concentrations of purified SiSV or RLV, which were disrupted by incubation with 0.3% Nonidet for 1 hr. The amount of viral protein was determined by the method of Lowry [8]. Linear regression analysis was applied to fluorescence values falling in the concentration-dependent range.

Sepharose bead immunofluorescence assay of antiviral antibodies

Sepharose beads were covalently coupled with disrupted virus. They were then incubated with inactivated (45 min, 56°C) rat sera. After repeated washing, they were incubated with a goat antiserum to rat IgG conjugated with FITC (Nordic Immunological Laboratories. Tilburg, The Netherlands).

RESULTS

Figure 2 shows the fluorescence found with different dilutions of SKA21-3 tissue culture supernatant using an antiserum, directed against the p28 protein of SiSV, bound to Sepharose beads. Supernatants of uninfected SIRC and RLV-producing BALB/3T3 cells were used as controls. The protein concentration was adjusted to 10 mg/ml for each culture fluid. The antiserum directed against SiSV-p28 cross reacts with purified RLV only when the concentration of RLV protein is higher than 1700 μg per ml. The initial concentration of the SiSV preparation was 0.6 mg/ml. From Fig. 2, it can be roughly estimated that the SKA21-3 supernatant contained approximately 1 μg SiSV related proteins per ml, since a 25-fold dilution of the culture fluid corresponds with roughly a 15,000 dilution of the purified virus.

A more detailed calibration curve was prepared to measure the amount of SiSV related viral proteins quantitatively. This calibration curve, depicted in Fig. 3, has a sigmoid shape with a background plateau at 30 and a saturation plateau at 1200 ng per ml of SiSV.

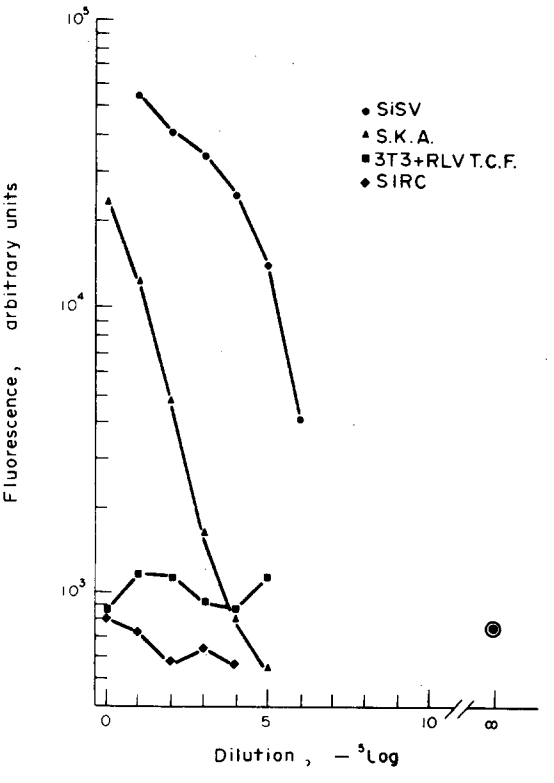

Fig. 2. Characterization of viral proteins in the culture fluid of SKA21-3. Sepharose beads coated with an immunoglobulin-G fraction of a goat serum directed against the p28 of simian sarcoma virus (Seph-GA SiSV/p28) were incubated with fivefold dilutions of samples of culture fluids of SKA21-3, SIRC and BALB/3T3 RLV and subsequently with GA SiSV p28 FITC. Each value represents the average fluorescence of at least five individual beads. The protein concentration of all undiluted samples was 10 mg/ml. *For comparison, a preparation of purified SiSV was used.*

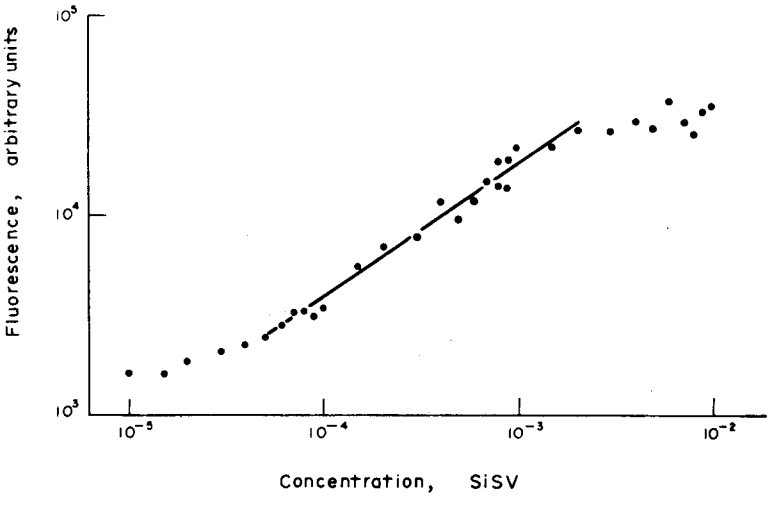

Fig. 3. Calibration curve for simian sarcoma viral antigens with Sepharose beads coupled with an immunoglobulin-G fraction of a goat serum directed against the p28 protein of simian sarcoma virus (Seph-GA SiSV/p28). The undiluted preparation contained 0.6 mg/ml *proteins. Seph-GA SiSV/p28 was incubated with different dilutions of the SiSV preparations and subsequently with GA SiSV/p28-FITC. Each value represents the average fluorescence of at least five individual beads. The equation for the solid line was calculated by the least squares method. The concentration of the undiluted sample was taken as* 1 *on the abscissa.*

The fluorescence is linearly dependent on the virus concentration between these two values. No reactivity of this antiserum was found with fetal calf serum, even at concentrations of 5 mg protein per ml.

The concentration of SiSV-related proteins in different experiments (Table 1) has been determined on the basis of the calibration curve. The A21 coculture of A7573 dog thymus cells and human leukemic bone marrow cells, which preceded the SKA21-3 line, was negative in the test. The amount of viral antigens in the supernatants of 2 different passages of the SKA21-3 culture was respectively 635 and 3320 ng per ml, while the concentration was considerably lower in supernatants of cultures infected with SiSV (73 and 151 ng/ml). The supernatant of the A204 line infected with SKA21-3, which did not produce focus-forming MSV, was also a high producer of a SiSV-related virus (767 ng/ml).

When lymph node cells of a rat with a SKA21-3 induced sarcoma was cocultivated with A7573 cells the culture became, after 3 weeks, a high producer of a C-type oncornavirus, as estimated by the reverse transcriptase assay. The Sepharose bead immunofluorescence assay indicated a concentration of 524 ng of SiSV-related proteins per ml culture fluid of this co-culture. The supernatant contained 80 focus forming units/ml when titrated on rat embryonic fibroblasts. The original SKA21-3 cultures produced 10^3 to 10^4 units/ml.

The coculture of A204 cells with lymph node cells of a rat in which leukemia had been induced by A204(SKA) virus also became a good virus

Table 1. Quantification of SiSV related antigens in tissue culture fluids

Tissue culture fluid	SiSV equivalents (ng/ml)
WAG/Rij rat embryonic fibroblasts infected with SiSV	151 ± 8*
NC37 suspension culture infected with SiSV	73 ± 6
SKA21-3 (a)	635 ± 84
SKA21-3 (b)	3332 ± 373
A204 (SKA)	767 ± 134
Sarcomatous lymph node + A7573	524 ± 94
Leukemic lymph node + A204	425 ± 86
RLV (1.7 mg/ml)	< 30
Fetal calf serum (24 mg/ml)	< 30

*av ± S.D. of quadriplo's.

producer after 4 passages. No focus-forming activity was detected in repeated assays on rat embryonic fibroblasts. The concentration of viral antigens determined with the antiserum to SiSV-p28 was 425 ng/ml. No reaction was observed with beads coupled with the antiserum directed against RLV (Fig. 4). SiSV related antigens were found also in the sera of three rats with leukemia induced by the MSV-free A204(SKA) virus. These sera contained no antibodies to either SiSV or RLV, whereas normal rats have a low titer of antibodies to SiSV and a considerably higher level of antibodies to RLV (Fig. 5).

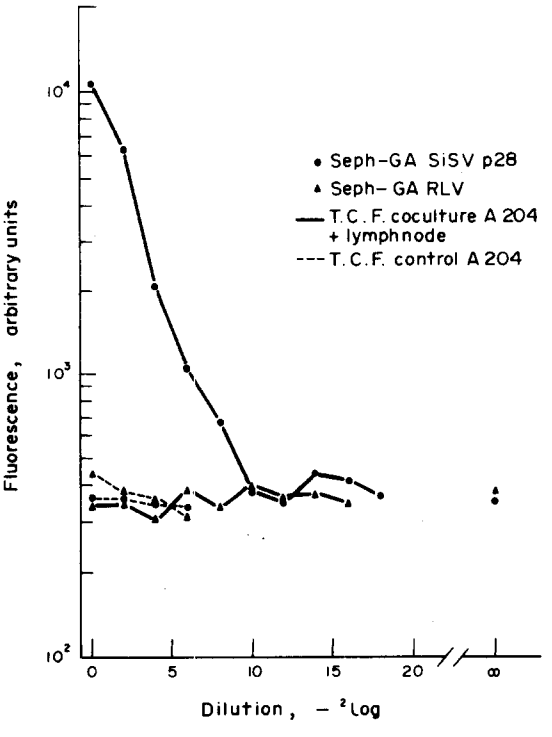

Fig. 4. Reactivity of culture fluid from cocultures of a leukemic lymph node and the human A204 line with beads coupled with an antiserum to Rauscher leukemia virus (Seph-GA RLV) or an antiserum directed against the p28 protein of simian sarcoma virus (Seph-GA SiSV/p28).

DISCUSSION

The Sepharose bead immunofluorescence assay (SBIA), developed for the measurement of immunoglobulins, can be successfully applied to the detection of oncornaviral proteins. As is the case with immunoglobulins [4, 5], the method proves to be reliable and accurate. The lowest concentration of viral antigens which can be detected with this method is 1.5 ng per sample of 50 μl. Such a sensitivity is comparable to that of the radioimmunoassay (RIA) which is widely used in tumor virology. The advantage of this new technique in comparison with RIA is the avoidance of false positives due to destruction of labeled antigens by proteolytic enzymes. Another advantage of the SBIA is that reagents can be stored indefinitely, while, in the RIA, the antigen must be iodinated, roughly, bimonthly.

The test, using antisera to purified viral polypeptides, proved to be specific, in that no

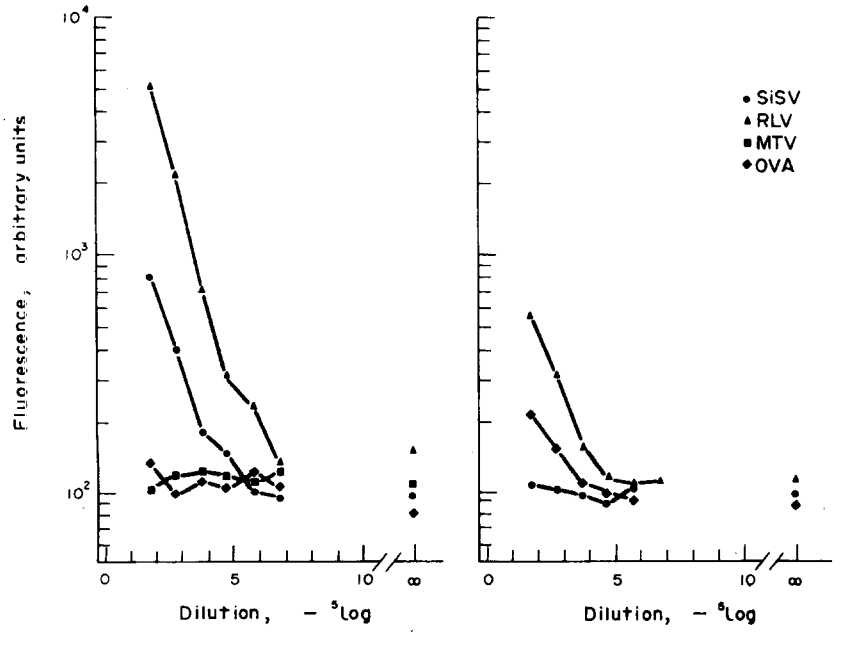

Fig. 5. Detection of antibody reactivity against SiSV and RLV in the serum of leukemic or normal rats using Sepharose beads coated with either virus.

reactions were found with culture fluids from uninfected cell lines or with (large amounts of) fetal calf serum. Only a very weak reaction of the antiserum to SiSV-p28 was found with high concentrations of Rauscher murine leukemia virus.

The high concentration of proteins antigenically related to SiSV-p28 in the SKA21-3 culture fluid indicates that the virus retrieved from human leukemic bone marrow cells is related to the simian sarcoma virus not only with regard to envelope antigens but also to the major internal polypeptide. Indirect immunofluorescence studies on acetone-fixed cells using a polyvalent antiserum to SiSV had already given an indication that more than the envelope proteins of the SKA21-3 virus would be antigenically related to SiSV [2].

The A21 coculture, which preceeded the SKA21-3 line, was negative with the SB1A. The reverse transcriptase values were low, while cytoplasmic immunofluorescence was clearly positive for SiSV. Probably there was considerable synthesis of internal viral proteins, but little release of complete virions. The recovery of an SiSV-related virus from a tumorous lymph node of a rat injected with SKA21-3 indicates that the human helper virus can establish itself in rats.

The A204(SKA) virus, which according to the focus-forming test would not contain MSV, induces lymphosarcomas in rats. The virus, retrieved from leukemic rats of a tumorous lymph node by cocultivation with A204 cells, is also related to SiSV and, in addition, does not contain MSV, as no focus-forming activity was found *in vitro*. The lymphosarcoma does not seem to be induced by MSV. Since no virus related to RLV has been detected in the tumor, it is unlikely that the disease was induced by mere activation of an endogenous rat virus. So far, we have failed to detect close antigenic relationship between SiSV and endogenous rat viruses released either spontaneously or after induction with halogenated pyrimidines. These rat viruses show considerable antigenic cross-reactivity with RLV. It seems therefore that the human-derived helper virus is itself leukemogenic in rats. It is an attractive hypothesis that a SiSV-related virus is also leukemogenic in man [9, 10].

A remarkable finding was the absence of antibodies in the leukemic rats, while normal WAG/rats have a low but significant titer of antibodies reacting with SiSV. Normal rat sera have a much higher specific reactivity with RLV than with SiSV and it is assumed that the natural antibodies are directed against an endogenous rat virus which is more closely related to RLV than SiSV (G. Koch, manuscript in preparation). It is to be expected, however, that infection with SiSV would enhance the reaction against determinants which the endogenous rat virus would have in common with SiSV. The absence of a reactivity with both RLV and SiSV could be due to either immune complexes or to immunosuppression. The latter can be explained by replacement of normal lymphoid elements by leukemic cells. However, several leukemia viruses have an immunosuppressive effect long before the onset of the neoplastic disease (for review, see [11, 12]).

In man, antibodies to SiSV seem to be widespread [13, 14]. However, the titer of such antibodies is low or they are virtually absent in leukemic patients [13]. The lymphosarcoma induced in rats by a human-derived SiSV-related virus might therefore be a relevant model for the human disease.

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Possible Relationship of Plasma IgA, IgG and IgM to Breast Cancer in British and Japanese Women

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Abstract—*Plasma levels of IgA, IgG and IgM have been measured in 35 British and 37 Japanese normal adult women, in 22 British and 22 Japanese adolescent girls and in 30 Japanese women with breast cancer.*

Japanese women had significantly higher blood concentrations of IgG and IgM than British women. The amounts of plasma immunoglobulins in adolescent British and Japanese girls were similar to those in adults of the same race, except for IgG, where Japanese adolescent girls had significantly lower levels than in adult Japanese women.

In contrast to previous results showing no difference between the IgA, IgG and IgM levels of normal British women and patients with breast cancer, the IgM concentration was significantly lower in the Japanese patients compared with that in Japanese controls.

INTRODUCTION

WE HAVE been investigating various factors that might be related to geographical variation in the incidence of breast cancer. So far we have been unable to find any convincing difference in the endocrine function of normal British and Japanese women that could account for the marked difference in incidence [1–3], but 57% of Japanese patients with breast cancer have been shown to have a moderate or marked degree of sinus histiocytosis in their lymph nodes compared to only 4% in British patients [4]. Also the degree of lymphocyte infiltration is greater in tumours from Japanese patients than in those from Caucasian women [5].

These findings indicate a racial difference in cell-mediated response to breast cancer and prompted the investigation of humoral immune status by determining the concentration of plasma IgA, IgG and IgM in normal British and Japanese women and in Japanese patients with breast cancer.

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MATERIAL AND METHODS

Subjects

The general organization of the study and details of height, weight and menstrual history of the normal women have been reported [1].

The number, mean age and age range of the various groups studied are given in Table 1. The 30 Japanese and 22 British women with breast cancer had either Stage 1 or 2 disease. Blood was taken from these women 3–6 months after mastectomy.

Methods

The immunoglobulins IgA, IgG and IgM were measured by rocket immunoelectrophoresis [6].

Standards for IgA, IgG and IgM were commercially obtained from Millipore (UK) Ltd. In a previous publication [7] it has been demonstrated that the strength of these standards using WHO figures (1 i.u. is equivalent to 14.2 μg IgA, 80.4 μg IgG and 8.47 μg IgM) was remarkably similar to the potency obtained when they were compared to the MRC reference

Table 1. Number and age of women studied

Category	No.	Mean age	Age range
British adolescents	22	17.3	16–
Japanese adolescents	22	18.7	16–19
British adults	35	52.7	35–68
Japanese adults	37	49.6	35–66
British patients with breast cancer	22	56.6	44–68
Japanese patients with breast cancer	30	50.9	37–64

preparation (67/99) of IgA, IgG and IgM [7]. Thus all results have been expressed as i.u./ml as advocated by WHO.

Included in each batch of 8 samples analysed was a quality control plasma. If the results for the quality control were greater than 15% from the mean value of IgA, IgG or IgM, the batch of samples was assayed again.

Precision of the assay as calculated by comparing duplicate estimations showed that the coefficient of variations, in the range studied, was 12.6, 6.4 and 5.3% for IgA, IgG and IgM respectively [7].

RESULTS

1. *Normal British and Japanese women*

IgA. There was no statistical difference between the geometric mean level of plasma IgA in normal British and Japanese women nor did the level found in the adolescent girls differ from those found in the adults (see Table 2; Fig. 1).

IgG. The geometric mean plasma level of IgG was significantly higher in Japanese women than in British women ($t=3.4$; $P<0.01$; d.f. $=70$). The IgG levels in adolescent British girls were similar to those found in adults but in Japanese adolescents the levels were significantly lower than those in older women ($t=2.20$; $P<0.05$; d.f. $=57$). Even so, the average concentration in Japanese adolescents was significantly higher than in comparable British girls ($t=4.05$; $P<0.001$; d.f. $=42$).

IgM. The geometric mean level of IgM was significantly higher in Japanese adult women compared with that found in the British group ($t=5.2$; $P<0.001$; d.f. $=68$). The IgM levels in adolescent girls of each race resembled those found in the adults; that is to say, the IgM levels were higher in the Japanese adolescents than in the British.

2. *Patients with breast cancer*

The geometric mean levels of IgA and IgG in Japanese patients with breast cancer are comparable to those in normal Japanese women but the IgM levels in the patients were significantly sub-normal ($t=2.9$; $P<0.01$; d.f. $=61$). The IgM level in the Japanese women with breast cancer (131 i.u./ml) is similar to that found both in British patients (151 i.u./ml) and in normal British women (124 i.u./ml) [7]. The results are shown in Fig. 2.

In British women, the mean levels of IgA, IgG

Table 2. Immunoglobulin levels in British and Japanese women

	IgA	IgG	IgM
British adolescent	2.06 ± 0.19 (22)	2.09 ± 0.10 (22)	2.17 ± 0.18 (16)
Japanese adolescent	2.12 ± 0.17 (21)	2.20 ± 0.08 (22)	2.23 ± 0.22 (19)
British adult	2.10 ± 0.21 (33)	2.13 ± 0.11 (35)	2.09 ± 0.23 (35)
Japanese adult	2.16 ± 0.15 (36)	2.26 ± 0.11 (37)	2.26 ± 0.17 (35)
British breast cancer	2.04 ± 0.22 (21)	2.07 ± 0.25 (21)	2.18 ± 0.22 (19)
Japanese breast cancer	2.15 ± 0.16 (30)	2.23 ± 0.10 (30)	2.12 ± 0.22 (28)

All results expressed as mean $\log_{10}$ i.u./ml $\pm$ S.D.
The numbers in parentheses refer to the number of subjects studied.

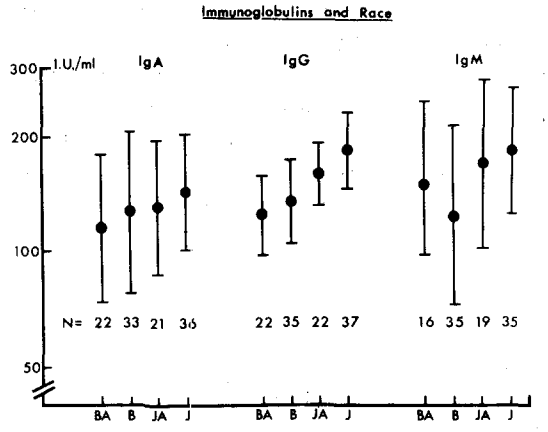

Fig. 1. Plasma immunoglobulin levels and race. BA and JA represent British and Japanese adolescents, respectively. B and J represent British and Japanese adults. N is the number of subjects in each group and the results are expressed as mean ± standard deviation.

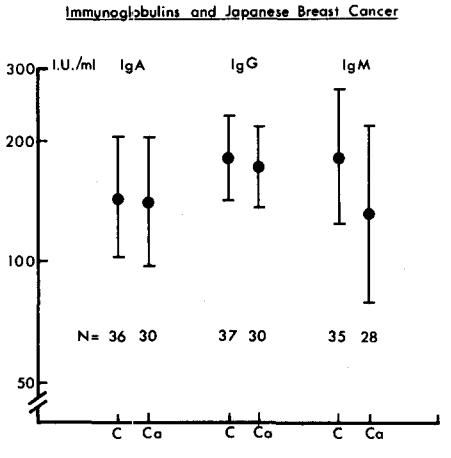

Fig. 2. Plasma immunoglobulin levels in normal Japanese women and patients with breast cancer. C and Ca represent Japanese normal control and breast cancer patients, respectively. N is the number of subjects in each group and the results are expressed as mean ± standard deviation.

and IgM do not differ significantly when normal women and patients with breast cancer are compared [7].

DISCUSSION

The amounts of IgA in the plasma were similar in both Japanese and British women, with and without breast cancer. In contrast IgM and IgG are significantly higher in normal Japanese women than in British women, in all age ranges studied. Although studies in twins indicate that levels of IgM and IgG are partially genetically determined [8, 9] the main factors affecting the concentration of these compounds in blood are environmental [10].

We have already reported that IgG levels are normal in British women with breast cancer [7] and this is similar to the finding of a normal IgG concentration in Japanese patients with breast cancer. Conversely, the similarity in IgM levels found by many workers [7, 11–15] in Caucasian women with and without breast cancer is not seen when normal Japanese women are compared to their compatriots with breast cancer; the latter have significantly subnormal levels of IgM.

It would be an attractive hypothesis that this abnormality is IgM levels in Japanese patients with breast cancer is associated with the aetiology of the disease. The fact that Caucasian women with breast cancer have been found to have normal levels of IgM one day before, 10–14 days or 3–6 months after mastectomy makes it unlikely that the abnormal levels in Japanese patients are a consequence of either the disease or its treatment [7].

There is evidence that abnormalities of humoral immunity are associated with increased incidence of neoplasms but these tend to occur mainly in lymphoid tissue [16]. However, common variable primary immunodeficiency (also referred to as acquired agammaglobulinaemia) is a disorder in which most individuals have abnormalities of both B and T cells. Of these patients who develop cancers, 39% are of epithelial tissue including breast [16]. Although there is no evidence linking IgM with the aetiology of any human cancer there is a body of evidence which connects IgM with tumorigenesis in the nude mouse.

Jensen and Wellings [17] have remarked on the lack of carcinomatous transformation of transplanted atypical lobules (type A) in nude mice and Schmidt and Good [18] have commented that human tumours with a high propensity to metastasize generally fail to do so in these animals. These authors have suggested that the residual immune system in this animal is responsible and it is significant that this appears to be mainly humorally mediated by normal levels of IgM since levels of IgA and IgG are only some 15% of those found in normal mice [19, 20]. T cell function appears to be minimal in nude mice since thymus rudiments transplanted into normal mice fail to support thymocyte proliferation [21].

It is also of interest that IgM synthesis is less dependent on thymus tissue than IgG and IgA production and this may account for the single abnormality found in Japanese patients with breast cancer [22, 23]. Thus the results could be interpreted to mean that Japanese women with breast cancer have a normally functioning

thymus producing a normal T cell population, IgA and IgG levels, but that the independent production of IgM is impaired.

In a comparative study concerned with the endocrine status of Japanese and British women the main finding was a lower plasma cortisol (unpublished observations), dehydroepiandrosterone sulphate and androstenedione [3]. However, the inter-racial differences in immunoglobulins do not seem to be related in any obvious way with these endocrine differences since the concentration of none of these hormones is significantly correlated, in either race, with the levels of any of the immunoglobulins.

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Dimethyl Myleran and Autologous Marrow Grafting for the Treatment of Spontaneous Canine Lymphoma*

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Abstract—*Dimethyl myleran (DMM), an antitumor drug effective against several rodent and human tumors, has severe hematopoietic toxicity, but only moderate immunosuppressive activity in dogs. In the present study,* 18 *dogs with spontaneous lymphoma were treated with* 7.5–10 mg/kg *DMM followed by infusion of previously aspirated autologous marrow to protect against otherwise lethal hematopoietic toxicity. Eleven dogs died in less than* 14 days, *several of unexpectedly severe gastrointestinal toxicity. Seven dogs survived* 15–95 (*median* 38) days. *Only* 1 *dog achieved a transient complete clinical remission;* 12 *additional dogs showed a greater than* 50% *decrease in palpable adenopathy. All but* 1 *dog had histologic evidence of lymphoma at autopsy.*

Immune responses were studied in 6 *dogs surviving greater than* 30 days. *Antibody formation to sheep red blood cells was delayed, but ultimately titers were similar to those of normal dogs. Production of lymphocytotoxic antibody was slightly enhanced while production of antibody to bacteriophage was slightly impaired. Although high dose DMM alone does not appear to be effective in achieving complete remission in dogs with lymphoma, the relative lack of immunosuppression following DMM preserves the potential usefulness of this drug in the design of chemo-immunotherapy protocols.*

INTRODUCTION

MUCH attention has been devoted in both animal and human studies to the efficacy of active immunotherapy in the treatment of neoplastic disease [1, 2]. In general, an attempt is made to stimulate the immune reactivity of the tumor bearing individual by administration of either specific or non-specific agents. Frequently, however, such attempts may be impaired by concurrent administration of cytotoxic chemo- or radiotherapy. Such therapy is utilized in order to decrease the tumor burden, but unfortunately is usually also immunosuppressive. Design of active chemo- or radioimmunotherapy protocols would be facilitated by the availability of effective antitumor agents which were not immunosuppressive.

Dimethyl myleran (DMM), a more soluble and potent homologue of busulfan (Myleran®), is an agent with little immunosuppressive activity in rodents [3, 4] or dogs [5] but significant antitumor activity against Moloney lymphoma in mice [6] and granulocytic leukemia [7] and anaplastic carcinoma of the postnasal space in man [8]. However, the marked myelosuppressive activity of DMM has limited its clinical usefulness. In the current study, we treated dogs with naturally occurring lymphoma with a high single dose of DMM. Since spontaneous bone marrow recovery was not seen in healthy normal dogs after similar doses of DMM [5], we attempted to prevent death from marrow aplasia by infusion of previously aspirated autologous marrow. Tumor response and immune reactivity were assessed following DMM and marrow infusion.

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MATERIAL AND METHODS

Procedures for procurement of dogs, initial evaluation, aspiration of marrow for autologous grafts, administration of DMM, post-grafting care and immune evaluation have all been previously published in detail [5, 9, 10] and will be only briefly summarized. Dogs of various breeds with generalized adenopathy were referred by veterinarians with consent of their owners. Eleven dogs were male, 7 were female; average age was 6.7 yr. Diagnosis of lymphoma was confirmed by histological examination of a peripheral lymph node biopsy. Marrow involvement with tumor was assessed from a single marrow aspirate by examination of smears and sections of Zenker's fixed, centrifuged particle preparations. Tumor measurements—perpendicular diameters of submandibular, prescapular, popliteal and inguinal nodes—were recorded before and after therapy. Eleven dogs had received no known prior chemotherapy; 2 had been treated with 9 and 16 day courses of L-asparaginase, 250–300 units/kg and 6-mercaptopurine, 1 mg/kg; and 5 had received combination chemotherapy consisting of cyclophosphamide, 5 mg/kg and vincristine, 0.025 mg/kg, on days 1 and 8 and L-asparaginase, 250 mg/kg and prednisolone, 2 mg/kg, on days 1–14.

Before DMM administration, marrow was aspirated under general anesthesia from both humeri and femora as previously described [11]. A mean of 1.2 ± 0.9 (S.D.) $\times 10^8$ nucleated marrow cells/kg (after correction for peripheral blood "contamination") was obtained, kept at 4°C until 6 hr after DMM administration and then infused intravenously. In one dog, cryopreserved autologous marrow was utilized [11]. DMM, 7.5 mg/kg in 16 dogs and 10 mg/kg in 2 dogs, was dissolved in warm absolute ethanol and given slowly intravenously as a bolus immediately after marrow aspiration [5].

Parenteral fluids, antibiotics and transfusion support were administered as clinically indicated following DMM and marrow infusion. Humoral immune responses to sheep red blood cells (SRBC) given on day 0 (i.e., the day of DMM and marrow infusion), to bacteriophage ØX174 (phage) given on day 14 and to allogeneic leukocytes in blood transfusions given on days 5–49 as clinically indicated for platelet support were measured as previously described [5, 10]. Dogs were immunized with Bacille Calmette–Guerin (BCG) on day 21 and tested for cutaneous reactivity to second strength purified protein derivative (PPD) on day 42.

Statistical comparisons were made using a two-sided Mann–Whitney U test.

RESULTS

Survival

The median survival of lymphoma dogs following 7.5–10 mg DMM and autologous marrow infusion was 10 (range 2–95) days. Eleven dogs died in less than 14 days, generally of gram negative septicemia or pneumonia while severely leukopenic. Unexpectedly severe gastrointestinal toxicity contributed to the death of 5 dogs during the first 14 days. Seven dogs survived 15, 32, 33, 38, 40, 50 and 95 days. The nadirs of peripheral granulocyte counts occurred on days 4–9 (median 8) with counts ranging from 0 to 275 (median 23) granulocytes/mm^3. The nadirs of platelet counts occurred on days 8 and 12 in 2 dogs with subsequent increases in platelet counts; in 4 dogs platelet counts never increased to sustained values of greater than 10,000/mm^3 (1 dog dying on day 15 is not evaluable with regard to platelet recovery). Among the dogs living greater than 30 days, 1 died of late gastrointestinal toxicity (onset day 8) and 3 died with septicemia (2 beta-hemolytic streptococci, 1 clostridia sp.) in spite of adequate peripheral granulocyte counts.

Dogs living more than 14 days were compared to those living less than 14 days with regard to prior exposure to chemotherapy, overall clinical status, number of marrow cells/kg infused and presence or absence of malignant cells in pregrafting marrow aspirates or peripheral blood smears. No difference between the groups with regard to any of these characteristics could be detected.

Tumor response

The maximal therapeutic effect upon clinically palpable tumor following DMM is indicated in Table 1. In order to quantify tumor response, the average maximal decrease in the product of perpendicular node diameters was estimated. Complete remission indicated inability to palpate any peripheral nodes. Dogs with greater than 75% decrease in tumor had minimal residual palpable adenopathy; dogs with 50–75% decrease had large reductions in tumor mass but significant persistent adenopathy; and dogs with less than 50% decrease had only minimal antitumor effect. As is evident in Table 1, most dogs had substantial, but not complete decrease in palpable tumor after receiving DMM. There is a tendency towards improved survival in dogs with greater decrease in palpable tumor, but the small number of dogs does not permit a firm conclusion. Dogs surviving more than 30 days achieved maximal decrease in adenopathy 14 days following

Table 1. Clinical response among dogs with lymphoma following dimethyl myleran and autologous marrow grafts

Maximum decrease in palpable tumor*	Number of dogs	Survival > 14 days	
		Number of dogs	Days
Complete remission	1	1	95
> 75%	3	2	38, 40
50–75	9	3	15, 32, 50
< 50%	4	1	33
Not evaluable†	1	0	—

*See text for details.
†Died on day 2.

DMM; increasing adenopathy was evident in all by day 28.

At autopsy all dogs grossly appeared to have residual (or recurrent) tumor. It was often difficult to distinguish histologically between lymphoma and regeneration of normal lymphoid cells following chemotherapy. Monomorphous clusters of cells similar to those present in pretransplant biopsies were considered malignant. Seventeen dogs had histological evidence of lymphoma: tumor was present in 61 of 84 lymph nodes examined and in the liver in 10 dogs, in the spleen in 14 dogs, in the marrow in 6 dogs and in miscellaneous sites in 9 dogs. No definite histological evidence of tumor was found in a single dog that died 5 days following DMM with greater than 75% clinical decrease in tumor.

Immune reactivity

Results of studies in 6 dogs to assess humoral immune reactivity following DMM and autologous marrow grafting are presented in Table 2. Comparable data obtained in previous studies in lymphoma dogs and in normal dogs under various conditions are also presented in Table 2. Hemagglutinin titers to SRBC injected on day 0 are delayed in development, but by day 20 reach the level seen in normal dogs by day 8. The primary antibody response to phage was normal in 2 of 6 evaluable dogs and the geometric mean of the maximal antibody level was slightly higher than in lymphoma dogs before therapy or after total body irradiation (TBI) and autologous grafting. Nevertheless, the mean phage antibody titer was less than that observed in normal dogs. Two dogs each received 3 allo-

Table 2. Humoral immune reactivity following dimethyl myleran and autologous marrow grafts

Dogs						
Diagnosis	Treatment (reference)	Maximum No. of dogs studied	Antibody to SRBC*		Antibody to phage†	Lymphocytotoxic ab‡
			Day 8–12	Day 20		
Lymphoma	DMM and autologous marrow	6	3.0	6.3	0.30	5/6
Lymphoma	None [15]	27	2.7	ND§	0.21	4/7
Lymphoma	1200 R and autologous marrow [9]	6	1.1	2.4	0.11	2–5/6
Normal	DMM and autologous marrow [5]	15	6.3	7.3	0.24	9/15
Normal	None [10]	56	6.4	5.0	1.21	21/56
Normal	1200 R and marrow grafts [10]	33	4.5	3.9	0.21	14/26

*Increase in mean $\log_2$ titer.
†Geometric mean of maximal antibody activity following primary immunization, expressed as the rate of phage inactivation, or K value.
‡Number of dogs with complement dependent lymphocytotoxic antibodies/number of dogs tested.
§ND = not done.

geneic blood transfusions on days 7–13; 4 other dogs received 12–18 transfusions on days 5–49. One of the dogs receiving only 3 transfusions failed to make lymphocytotoxic antibodies while the other 5 dogs had detectable antibodies that appeared during the period of transfusion support.

Only 2 dogs lived long enough to be challenged with PPD following immunization with BCG. Both dogs had progressive adenopathy when tested on day 42 and failed to respond to PPD. No other tests of cellular immune reactivity were performed.

DISCUSSION

DMM appears to be more poorly tolerated by dogs with lymphoma than by normal dogs. Normal dogs receiving DMM, 3.0 or 5.0 mg/kg, experienced relatively prolonged granulocytopenia and thrombocytopenia, but ultimately recovered [5]. Higher doses (7.5 mg/kg) resulted in marrow aplasia and death but this could be averted by infusion of autologous marrow in 15 of 18 normal dogs given 7.5–10 mg DMM/kg. Following 15 mg/kg, however, 3 of 4 normal dogs succumbed secondary to severe enteritis in spite of autologous marrow infusion. In contrast, following 7.5–10 mg DMM and infusion of autologous marrow, only 7 of 18 dogs with lymphoma survived more than 14 days. Severe gastrointestinal toxicity was observed in 5 lymphoma dogs after 7.5 mg DMM/kg, i.e. at one-half of the dose required to produce comparable toxicity in normal dogs. Potential explanations for the increased toxicity observed include the age of the lymphoma dogs (mean 6.7 yr); their larger size (mean weight = 24.7 kg) and diverse breeds compared to the normal dogs studied (generally 6–14 month old, approximately 10 kg beagles); the effects of their underlying disease, previous chemotherapy and/or surgery and the lower number of marrow cells infused ($1.2 \pm 0.9 \times 10^8$/kg vs 4.5 $\pm 3.7 \times 10^8$/kg in normal dogs) [5]. Similar observations, i.e. increased toxicity in lymphoma dogs compared to normal dogs, have been previously reported following TBI and autologous [9] or allogeneic [12, 13] marrow grafting. Loss of a sizeable proportion of dogs in the first 2 weeks after drug administration and marrow infusion, however, still permits observations about the antitumor effect of DMM and studies of immune reactivity in dogs surviving more than 2 weeks.

In rodent studies, single high doses of DMM followed by normal syngeneic hematopoietic cells resulted in long-term, tumor-free survival in 51% of CBA mice bearing widespread Moloney lymphoma [6]. However, half the lethal dose was ineffective and neither lethal nor sublethal doses of DMM had significant antitumor effect against either Friend or Rauscher virus induced leukemias in C57Bl/6 mice [4]. DMM does have antitumor activity against canine lymphoma when utilized in maximally tolerated single doses followed by autologous marrow infusion (Table 1). Thirteen of 17 evaluable dogs had greater than 50% decrease in clinically palpable adenopathy. Complete remission, however, was achieved in only 1 dog and responses were transient. For comparison, TBI, in maximally tolerated single exposures, appears to be a more effective antitumor agent in this system. TBI resulted in greater than 75% decrease in palpable adenopathy in 12 of 17 evaluable dogs and complete remissions in 5 of these 12 [9]. Even though responses to TBI were also transient, median survival of dogs living more than 14 days was somewhat longer than following DMM (70 days vs 38 days, $P \sim 0.10$).

The results of the current study again emphasize the difficulty of correlating clinical and pathologic evaluation of tumor response to therapy [9]. One dog died with clinically palpable peripheral lymph nodes consistent with residual tumor, but histologically had lymphoid depletion, dilated sinusoids filled with granular debris and no definite evidence of tumor. Thus the tumor responses to DMM reported in Table 1 may underestimate the antitumor activity of the drug.

Our previous studies in normal dogs indicated that at doses of 3–10 mg/kg, DMM is only weakly immunosuppressive [5]. Antibody responses to SRBC and allogeneic histocompatibility antigens were normal while antibody responses to phage were mildly suppressed by 7.5–10 mg/kg. Cellular immune reactivity was not sufficiently suppressed by DMM to permit engraftment of incompatible allogeneic marrow in either mice [4] or dogs [5]. As indicated in Table 2, lymphoma dogs had little suppression of humoral immune reactivity by DMM. Responses were equal or greater than those in lymphoma dogs without therapy and appear to be superior to responses of lymphoma dogs following 1200 R TBI and autologous marrow grafting [9]. Unfortunately, too few tests are available to assess cellular immune reactivity.

The current study indicates that DMM has some, but limited, antitumor activity against spontaneous canine lymphoma. Although DMM should therefore not be the primary modality to minimize tumor burden prior to initiating active immunotherapy regimens, its

impressive lack of immunosuppressive activity may make DMM a useful agent in the design of such regimens. More frequent administration of lower doses might also result in less toxicity and obviate the need for autologous marrow aspiration and infusion.

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Tumorigenicity of Neuroblastoma × Glioma Hybrid Cells in Nude Mice and Reintroduction of Transplanted Cells into Culture

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Abstract—*Hybrid cells derived from mouse neuroblastoma line N18TG2 and rat glioma line C6-BU-1 are capable of generating tumors in athymic nude mice at 100% incidence. This fact provides a simple method for producing large quantities of cells needed for the biochemical characterization of some neuronal markers of the hybrid cells. Choline acetyltransferase specific activity, cloning efficiency, morphological differentiation and electrophysiological characteristics were similar for inoculated and tumor cells.*

INTRODUCTION

HYBRID cells obtained by fusion of mouse neuroblastoma line N18TG2 with rat glioma line C6-BU-1 display several neuronal functions including receptors for neurohormones and opiates [1–3]. These cells offer the possibility for further biochemical studies provided sufficient amounts of cells are available. One appropriate method of mass production of tumorigenic cells is the formation of tumors from cells injected into congenic animals. However, hybrid cells usually express the surface antigens of both parental cell lines [4]. Accordingly, the interspecific neuroblastoma × glioma hybrid cells were not expected to grow at high incidence in either rats or mice. Here we like to report the production and partial characterization of a tumor arising from interspecific neuroblastoma × glioma hybrid cells injected into athymic nude mice.

MATERIAL AND METHODS

Animals

The breeding and maintenance of athymic nude mice with the BALB/c genetic background has been described [5]. All animals were obtained from the animals house of the Max-Planck-Institut.

Cells

C6-BU-1 is a bromodeoxyuridine resistant rat glioma cell line [6]. N18TG2 is a 6-thioguanine resistant neuroblastoma cell line [7] derived from mouse neuroblastoma C1300. The formation of the hybrid cells 108CC5 and their culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), hypoxanthine (H) (0.1 mM), aminopterin (A) (10 μM) and thymidine (T) (16 μM) have been described [1–3]. Three days prior to the injection of the hybrid cells they were incubated in DMEM containing 10% FCS, (H) and (T) but not (A).

Production of tumors

After trypsinization, 1 million viable hybrid cells 108CC5 (P21) suspended in 0.2 ml DMEM were injected subcutaneously or intracerebrally into 3–4 weeks old nude mice. The passage number P designates the number of subcultivations.

Reintroduction of hybrid cells into culture and cloning

After mincing, a tumor grown intracerebrally, was dissociated into single cells by incubation with trypsin (0.05%) at 37°C for 5–20 min. After plating, the cells were cultured for 13 days in DMEM + 10% FCS and then seeded at low density. Three weeks later 10 colony clones from 10 different plates (designated 108CC5-T-1 PO

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Table 1. Tumorigenicity of neuroblastoma × glioma hybrid cells and of their parental cells, subcutaneously inoculated into rats and mice. Animals were tested for the production of tumors up to 10 weeks

Cell line injected	Number of animals with takes/total number of animals		
	Wistar rats	Strain A mice	Nude mice BALB/c
108CC5	0/15	0/10	25/25*
N18TG2	0/10	—	10/10
C6-BU-1	10/10	—	10/10

*A 100% take incidence is also found for hybrid cells injected intracerebrally.

Table 2. Specific CAT activity of hybrid cells, of tumors derived therefrom, and of hybrid clones reisolated from a tumor

Cell material	Designation of clone	Number of passages	Number of passages after reintroduction into culture	CAT specific activity pmole/min/mg protein
Culture	108CC5*	13	—	360§
Culture	108CC5*	70	—	120§
Tumor	108CC5†	16	—	120§
Tumor	108CC5-T2*	21 (before	12	136‡
reintroduced	108CC5-T4*	21 injection	8	145‡
into	108CC5-T6*	21 into	9	68‡
culture	108CC5-T8*	21 mice)	9	180‡

*Five days after plating of 1×10^6 cells on plastic dishes (85 mm dia), cell homogenates were prepared and specific CAT activity was determined.

†Tumor homogenates were prepared by trituration of 15 days old tumors with a Pasteur pipette followed by sonication as done with the cell culture samples.

‡§Values are averages from duplicate‡ and triplicate§ samples (plates or tumors).

to 108CC5-T-10 PO) were picked with the aid of stainless steel cylinders.

Characterization of cellular markers

Chromosomes were analysed as described [6]. Harvesting of cells, cell homogenisation and assays for choline acetyltransferase (CAT) (E.C.2.3.1.6.) activity were carried out as described [8]. Intracellular recording of action potentials from single cells in a superfusion chamber was done with standard techniques [9].

RESULTS

A 100% tumor incidence was found for the hybrid cells injected into nude mice (Table 1), whereas no tumor was produced in Wistar rats and strain A mice. The tumors in nude mice showed high specific activity of CAT (Table 2). Results obtained from an electronmicroscopic investigation of the tumors will be published elsewhere [10]. Also the parental neuroblastoma and glioma cells caused tumors in all nude mice inoculated.

After formation of intracerebral tumors, hybrid cells were reintroduced into culture (cells 108CC5-T) and their properties were compared with those of the original cells (108CC5). CAT specific activity (Table 2), cloning efficiency (10%, if 2 cells per cm^2 are seeded) and growth rate (data not shown) were seen to be in the range found with the original hybrid cells. Both rat and mouse marker chromosomes were present in the 108CC5-T cells, but the exact number of chromosomes was not determined.

One prominent neuronal quality of the hybrid cells, the ability to fire action potentials, was investigated in the tumor clones. For this purpose the cells were differentiated morphologically by treatment with N^6, $O^{2'}$-dibutyryl adenosine 3′ : 5′-cyclic monophosphate, as described earlier [11]. Upon electrical stimulation these cells generated action potentials (16 out of 20 cells measured). Upon elevation of the Ca^{2+} con-

centration in the medium, repetitive firing was observed and the action potentials could no longer be blocked by tetrodotoxin. Thus, the characteristics of the electrical activity were similar to those found in the original hybrid cells [9].

DISCUSSION

The results show that interspecific hybrid cells produce tumors in nude mice at a 100% take incidence. The cellular markers studied in culture before and after animal passages served as indicators for the retention of neuronal functions in the hybrid cell tumor. Growth characteristics, electrophysiological behavior, morphological differentiation and CAT specific activity of the hybrid cells isolated from the tumors were similar to those of the cells used for the injection into the mice. Except for subclone 108CC5-T6, the specific activity of CAT in the hybrid subclones (isolated from a tumor) was in the range of the original hybrid cells whose activity was measured at low (P13) and high (P70) passage numbers (Table 2). The observed variability of the specific activity of CAT in the hybrid subclones might be explained by the appearance of probably many subpopulations of hybrid cells due to the loss of chromosomes from the hybrid cells at low passage numbers [12].

In congruence with the results on hybrid tumor cells reintroduced into culture, high specific activity of CAT was also found in the tumor itself. Moreover, the tumor has a high capacity for specific binding of opiates [13].

It is not self-evident that cellular markers are preserved in cells derived from tumors. After heterotransplantation into nude mice ultrastructural and biochemical changes were found in cultured human melanoma cells [14]. In the present case, however, another [15] example is given for the constancy of cellular properties after passage through nude mice.

The tumorigenicity of intraspecific hybrid cells has been studied by others previously [16]. By fusion of different malignant mouse cell lines it was demonstrated that malignancy is suppressed in hybrid cells if one of the parental cell lines is nonmalignant. However, intraspecific hybrid cells arising from two malignant cell lines were as tumorigenic as the parental cells. Thus malignancy behaved as a recessive lesion at a single gene locus in mouse hybrid cells [17]. Our results of tumorigenicity of the interspecific neuroblastoma × glioma hybrid cells are in line with the above characterization of malignancy, since both parental cell lines N18TG2 and C6-BU-1 are subclones from transplantable tumors [18, 19]. Non-transformed cells such as primary tissue explants do not produce tumors in athymic nude mice [5, 20]. Moreover, it has been shown recently, that tumorigenicity in nude mice seems to be the most reliable assay for transformation [21].

For two reasons tumors from neuroblastoma × glioma hybrid cells appear to be useful tools in neuro-biochemistry. Firstly, characteristic differentiated functions are obviously preserved in the tumor cells. Secondly, the tumor weight in nude mice can nearly reach the body weight of the animal [15]. Therefore the large masses of hybrid cell protein can be produced easily, that are required, e.g. for the isolation of hybrid cell constituents such as CAT or receptors for neurohormones.

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Notes added in proof

In the meantime two papers from other laboratories appeared which show that hybrid cells are tumorigenic in nude mice.

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The Specificity of the Oestrogen Receptor of DMBA-induced Mammary Tumours of the Rat

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Abstract—*The comparative abilities of several naturally-occurring steroids to compete for [^3H]oestradiol-17β-binding sites in rat mammary tumour cytosol have been investigated. Apart from compounds containing a phenolic A-ring, the most effective compounds were 5-androstene-3β, 17β-diol, 5-androstene-3α, 17β-diol and 5α-androstane-3β, 17β-diol. 5-Androstene-3β, 17β-diol appears to compete by binding to the same site as oestradiol-17β. Those compounds most effective at diminishing cytoplasmic [^3H]oestradiol-17β binding also depressed nuclear binding of [^3H]oestradiol-17β. The presence of a nominal oestrogen receptor with a range of affinity for a number of steroids, including potential androgens, may indicate the presence of a regulatory mechanism superimposed on the basic oestrogen controlled system.*

INTRODUCTION

EVIDENCE which ascribes a role for androgens in the hitherto-designated oestrogen-target organs continues to accumulate. Certain of these tissues contain a discrete protein entity with definite selective affinity for androgenic steroids [1–3], while other reports [4, 5] suggest that the oestrogen receptor itself may sequester potential androgens to some extent. Previously [6], we have suggested that a macromolecule with varying ranges of affinity for steroids with different physiological roles may provide a regulatory system in the normal cell. Obviously, such a system achieves greater importance in the cancerous cell in view of its exploitation in therapy and, possibly, prognosis. This paper describes the study of the specificity of the cytoplasmic oestrogen receptors of mammary tumours induced in rats by 7,12-dimethylbenz[α]anthracene.

MATERIAL AND METHODS

Animals

Mammary tumours were induced in virgin female Sprague–Dawley rats (50 ± 2 days old) by intubations with a single dose of DMBA (20 mg in 1 ml sesame oil). Animals were housed in groups of 4 and allowed food and water *ad libitum*. Beginning 5 weeks after intubation with DMBA, rats were palpated for tumours at weekly intervals and tumour size was recorded as the mean of two perpendicular diameters, one measured across the greatest width. Tumours which did not attain an approximate size of 20 mm mean diameter in the 23-week period following carcinogen administration were not used in this study.

Preparation of subcellular fractions

Mammary tumours were homogenized [7] and cytoplasmic [8] and nuclear fractions [9] were prepared as previously described. The basic buffer system employed throughout comprised 50 mM-Tris–HCl buffer, pH 7.4, containing 5 mM-EDTA and 1 mM-dithiothreitol.

Detection of specific oestrogen-binding components in mammary tumour subcellular fractions

The labelling of subcellular fractions with [2, 4, 6, 7(*n*)-$^3H_4$] oestradiol-17β (specific radioactivity 85–110 Ci/mmole; Radiochemical Centre, Amersham, Bucks., U.K.), and subsequent sedimentation analysis has been adequately described [8, 10]. The quantitative assessment of receptor proteins selectively retaining [^{3}H]oestradiol-17β was performed by a charcoal adsorption technique [11] and analysis according to Scatchard [12]. The ability of certain other steroids to prevent the binding of

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[^{3}H]-oestradiol-17β to specific cytoplasmic receptors was examined by the following two methods:

(a) Constant amounts of cytosol (100 μl) were incubated with a saturating concentration of [^{3}H]oestradiol-17β (5 nmole/l) in the presence of varying concentrations of non-radioactive competitor (see 'Results'). After incubation (2 or 16 hr at 4°C) 200 μl of charcoal suspension [0.5% (w/v) Norit A, 0.05% (w/v) Dextran T-70, 0.1% (w/v) gelatin] was added. After removal of charcoal (800 *g* for 15 min), aliquots (100 μl) of the supernatant were assessed for bound radioactivity in 5 ml of scintillation fluid (5 g 2, 5-diphenyloxazole, 500 ml Triton X-100, 1 l. toluene) in a Nuclear Chicago (Mark II) liquid scintillation spectrometer at a counting efficiency for tritium of 30–40% as determined by external standardisation.

(b) Constant quantities of cytosol (100 μl) were incubated with three constant concentrations (0.18, 0.46 and 0.92 nmole/l) of [^{3}H]oestradiol-17β in the presence of varying concentrations of nonradioactive competitor (as detailed in the 'Results' section). After incubation (2 hr at 4°C), charcoal suspension (200 μl) was added and removed (as described above) and aliquots (100 μl) of supernatant were assessed for bound radioactivity. Data were expressed graphically in the form of single and double reciprocal plots.

Chemical analyses

The protein concentration of cytosol fractions was estimated using the method of Lowry *et al.* [13].

RESULTS

Characteristics of [^{3}H]oestradiol-17β-binding components in rat mammary tumour subcellular fractions

Sedimentation profiles of specific oestrogen receptor proteins from rat mammary tumour cytosol [9] and nuclei [10] have received adequate exposure. It is sufficient to reiterate that two species of [^{3}H]oestradiol-17β-binding proteins are found in cytosol, one of sedimentation coefficient 8S which shows specificity by accepted criteria and one of sedimentation coefficient 4S which comprises predominantly nonspecific components. Saturation analysis indicated the presence of a single class of high-affinity binding sites with dissociation constants in the range 50–1000 pmole/l and with concentrations in the range 10–200 fmole/mg protein. Those cytosols used in the competition studies had receptor concentrations >80 fmole/mg protein. Salt-extraction [10] of nuclei labelled with [^{3}H]oestradiol-17β yielded a single peak of protein-bound radioactivity on sucrose gradients corresponding to a sedimentation coefficient 4–5 S.

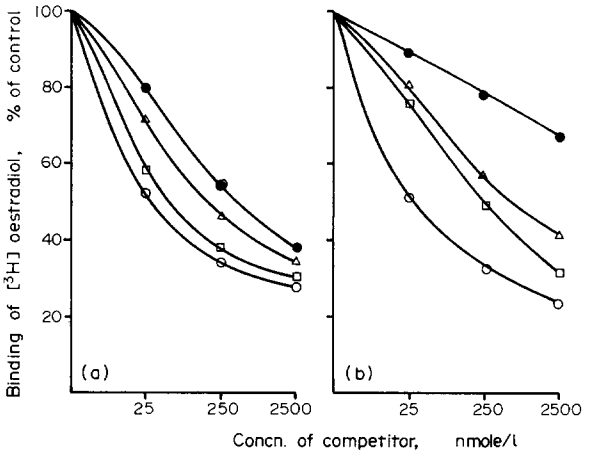

Fig. 1. Competition for [^{3}H]oestradiol-17β binding sites. Samples of cytosol were incubated with [^{3}H]oestradiol-17β (5 nmole/l) alone or in the presence of increasing concentrations of other steroids. Free and nonspecifically bound steroid was removed by charcoal adsorption and retained radioactivity was assessed by liquid scintillation spectrometry. Competing steroids were (a) oestradiol-17β (○), oestradiol-17α (□), oestriol (△) and oestrone (●); (b) oestradiol-17β (○), 5-androstene-3β, 17β-diol (□), 5-androstene-3α, 17β-diol (△) and 4-androstene-3β, 17β-diol (●). Binding is expressed as a percentage of charcoal-resistant binding in the presence of [^{3}H]oestradiol-17β alone (104 fmole/mg protein).

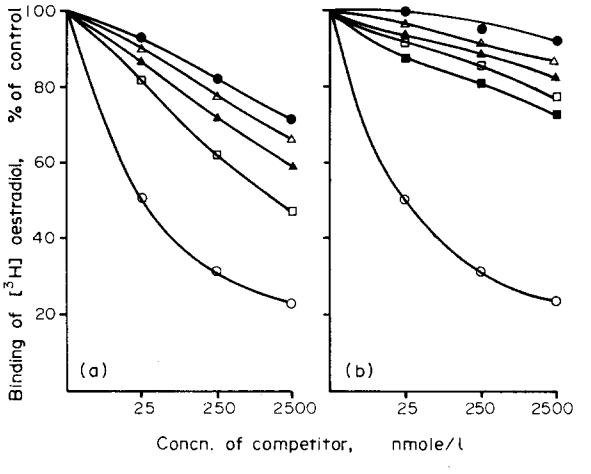

Fig. 2. Competition for oestradiol-17β binding sites. Details are essentially the same as for Fig. 1., but competing steroids were (a) oestradiol-17β (○), 5α-androstane-3β, 17β-diol (□), 5α-androstane-3α, 17α-diol (▲), 5α-androstane-3β, 17α-diol (△), 5α-androstane-3α, 17β-diol (●), and (b) oestradiol-17β (○), 5α-dihydrotestosterone (■), testosterone (□), androstanedione or epitestosterone (▲), dehydroepiandrosterone (△) and progesterone or corticosterone (●).

Competition for [^{3}H]oestradiol-17β-binding sites in mammary tumour cytosol

The comparative abilities of a number of steroids to compete with [^{3}H]oestradiol-17β for receptor sites is shown in Figs. 1 and 2.

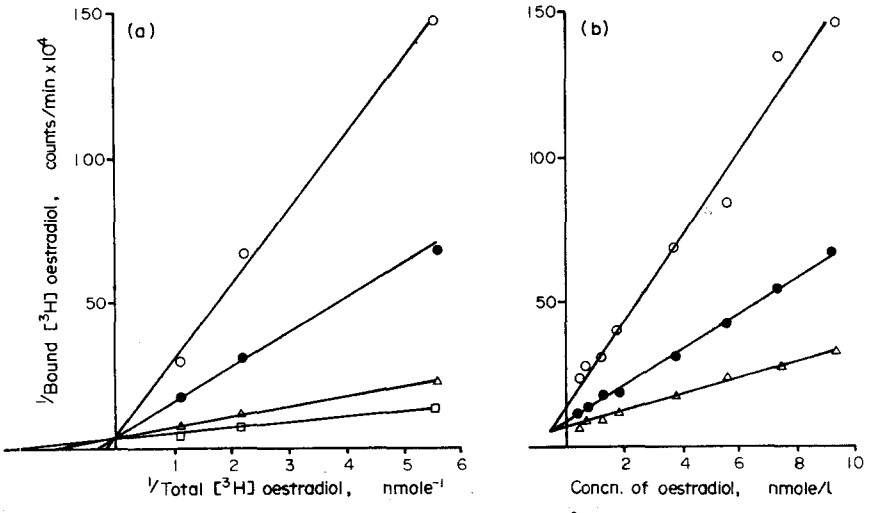

Fig. 3. Competition by unlabelled oestradiol-17β for specific [^3H]oestradiol-17β binding sites in mammary tumour cytosol. Aliquots (100 μl) of cytosol were incubated (2 hr at 4°C) with three constant concentrations of [^3H]oestradiol-17β (0.18, 0.46 and 0.92 nmole/l), alone or together with varying concentrations of oestradiol-17β in a total vol of 200 μl. After incubation, charcoal suspension (200 μl) was added and after 1 hr at 4°C was sedimented (800 g for 15 min) and aliquots (200 μl) of bound supernatant assessed for bound radioactivity. The concentrations of unlabelled oestradiol-17β used were 0.367 nmole/l (△), 3.67 nmole/l (●) and 9.19 nmole/l (○), (a) when the reciprocal values of bound [^3H]oestradiol-17β was plotted against the reciprocal value of total [^3H]oestradiol-17β. Control systems containing [^3H]steroid alone (□). (b) the reciprocal value obtained for bound [^3H]oestradiol-17β were plotted against total unlabelled oestradiol-17β concentrations, at concentrations of [^3H]oestradiol-17β of 0.18 nmole/l (○), 0.46 nmole/l (●) and 0.92 nmole/l (△).

Predictably, the most effective competitor was oestradiol-17β, together with oestradiol-17α (Fig. 1a). Oestriol and oestrone also competed very well (Fig. 1a). Of the three androstenediols studied, 5-androstene-3β, 17β-diol was the most effective compound, with 5-androstene-3α, 17β-diol able to reduce binding of [^{3}H]oestradiol-17β by 50–60% at the highest concentration used (Fig. 1b). 4-Androstene-3β, 17β-diol was less effective, causing a maximum decrease in specific binding of 30–40% (Fig. 1b). The 5α-androstanediols also showed variable potency, 5α-androstane-3β, 17β-diol being most effective (~55% decrease), followed by 5α-androstane-3α, 17α-diol (40%), 5α-androstane-3β, 17α-diol (35%) and 5α-androstane-3α, 17β-diol (~30%) (Fig. 2a). It must be emphasised that these degrees of competition are those achieved by the highest concentrations used i.e. 2500 nmole/l, or a 500-fold higher concentration than that of [^{3}H]oestradiol-17β. The compounds mentioned in Fig. 2b, therefore, can be regarded as relatively ineffective in decreasing the specific binding of [^{3}H]oestradiol-17β.

In view of the substantial reduction in [^{3}H]oestradiol-17β binding brought about by compounds such as 5-androstene-3β, 17β-diol it was thought worthwhile to test the hypothesis that these steroids are able to bind to the same site as oestradiol-17β, since competition could also be induced if the second steroid bound to an alternative site to alter protein conformation. This was done by monitoring competition under conditions of varying [^{3}H]oestradiol-17β concentration and varying concentrations of competing steroid (Figs. 3–6). Data obtained from competition studies using unlabelled oestradiol-

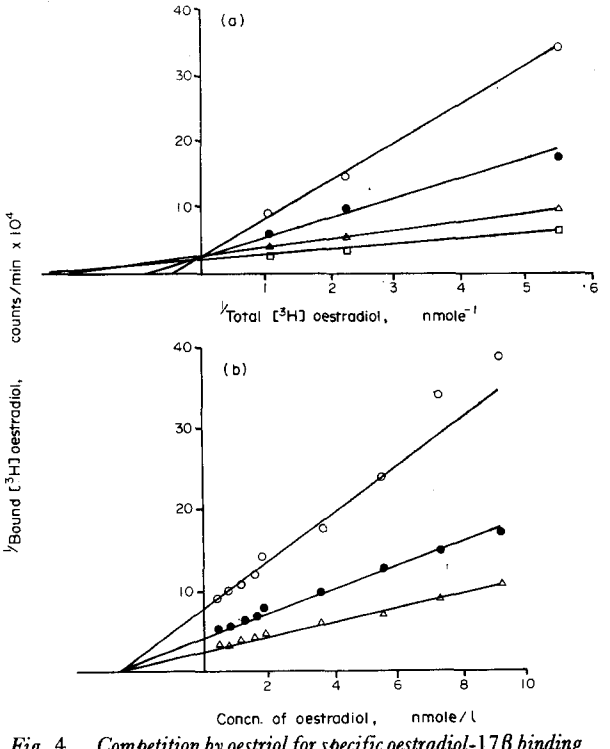

Fig. 4. Competition by oestriol for specific oestradiol-17β binding sites in mammary tumour cytosol. Details are essentially those in Fig. 3, except that oestriol was used as competitor. (a) [^3H]oestradiol-17β alone (□) and with oestriol at concentrations of 0.367 nmole/l (△), 3.67 nmole (●) and 7.35 nmole/l (○). (b) [^3H]oestradiol-17β at concentrations of 0.18 nmole (○), 0.46 nmole/l (●) and 0.92 nmole/l (△).

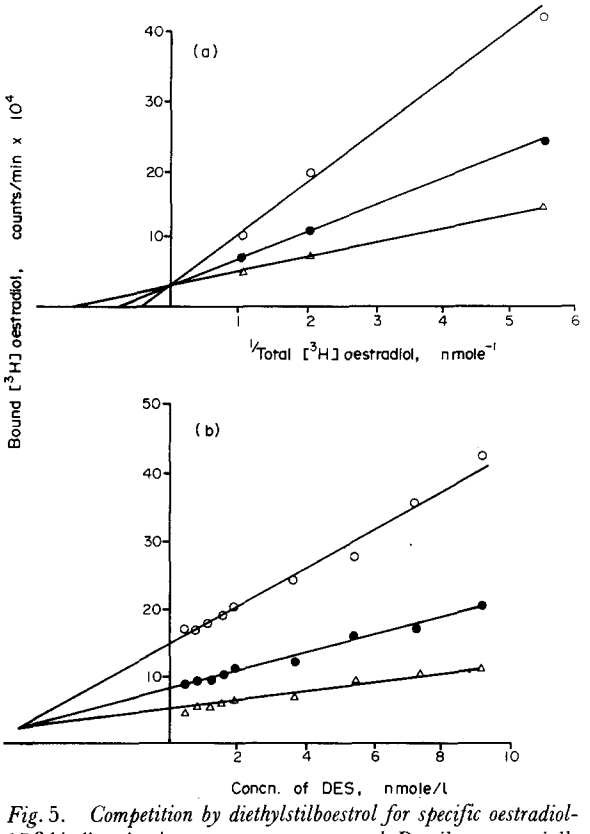

Fig. 5. Competition by diethylstilboestrol for specific oestradiol-17β binding sites in mammary tumour cytosol. Details are essentially those in Fig. 3, except that diethylstilboestrol was used as competitor. (a) [^{3}H]oestradiol-17β alone (△), or with diethylstilboestrol at 3.67 nmole/l (●) and 9.19 nmole/l (○) (b) [^{3}H]oestradiol-17β at 0.18 nmole/l (○), 0.46 nmole/l (●) and 0.92 nmole/l (△).

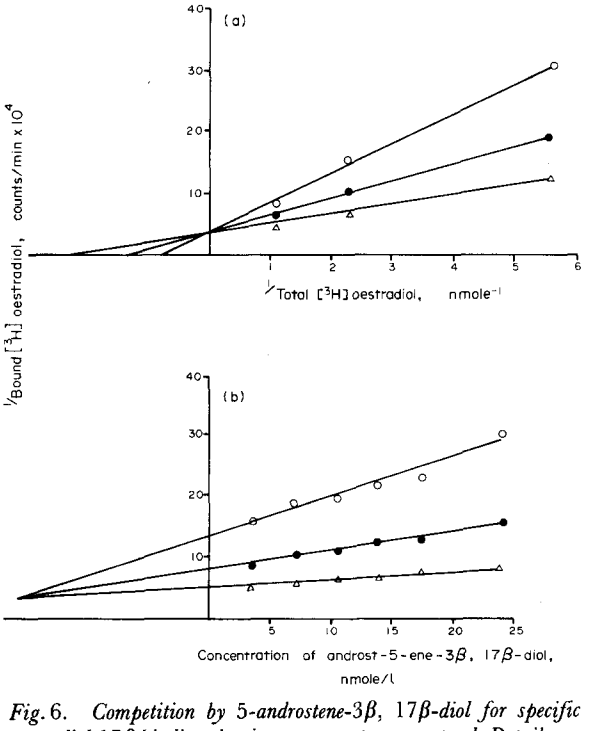

Fig. 6. Competition by 5-androstene-3β, 17β-diol for specific oestradiol-17β binding sites in mammary tumour cytosol. Details are essentially those in Fig. 3, except that 5-androstene-3β, 17β-diol was used as competitor. (a) [^{3}H]oestradiol-17β alone (△), or with 5-androstene-3β, 17β-diol at 10.30 nmole/l (●) or 25.86 nmole/l (○). (b) [^{3}H]oestradiol-17β at concentrations of 0.18 nmole/l (○), 0.46 nmole/l (●) or 0.92 nmole/l (△).

17β, oestriol (a steroidal oestrogen), diethylstilboestrol (a nonsteroidal oestrogen) and 5-androstene-3β, 17β-diol [the most effective of potential androgens in the competition studies (Fig. 1b)] as the competing substances were expressed graphically in the form of double reciprocal plots (Figs. 3(a)–6(a), respectively). The reciprocal value of bound [^{3}H]oestradiol-17β was plotted against the reciprocal value of total [^{3}H]oestradiol-17β. The patterns obtained were characteristic of competitive inhibition in that the presence of competitor altered the slope but not the intercept on the ordinate. To eliminate the possibility of allosteric inhibition, a plot of the reciprocal of bound radioactivity against the concentration of competitor was also used [Figs. 3(b)–6(b)]. Whereas allosteric inhibition would have resulted in hyperbolic curves, the substances under investigation produced a linear relationship indicative of direct competition. These data suggest that these compounds were competing with [^{3}H]oestradiol-17β for the same binding site.

Competition for oestrogen-binding sites in rat mammary tumour nuclei

Using methods described previously [10], the ability of various compounds to prevent the nuclear uptake of [^{3}H]oestradiol-17β was studied (see Fig. 7). A 1000-fold higher concentration of either oestradiol-17β or diethylstilboestrol completely depressed the 4-5S peak of radioactivity present in nuclear extracts after incubation with [^{3}H]oestradiol-17β alone, while similar concentrations of testosterone or 5α-dihydrotestosterone only slightly decreased the binding peak (Fig. 7). The inclusion of a 1000-fold higher concentration of 5-androstene-3β, 17β-diol or 5α-androstane-3β, 17β-diol resulted in a significant decrease in the binding of [^{3}H]oestradiol-17β (Fig. 7).

DISCUSSION

The ability of certain compounds to compete with [^{3}H]oestradiol-17β for specific binding sites has proved of great value in the development of anti-oestrogens [8, 14, 15], which may retard growth processes by impeding the obligatory retention step in mechanism of action of steroids. However, the possibility that naturally-occurring steroids may also compete for oestrogen-binding sites provides an attractive scenario for regulation of oestrogen action at the physiological level.

The data presented in Figs. 1 and 2 show that, apart from those compounds with a phenolic A-ring, i.e. naturally-occurring steroids with vary-

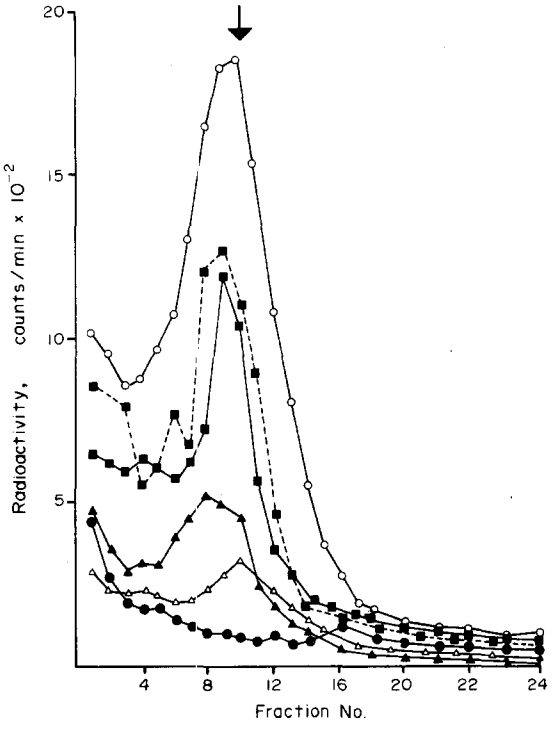

Fig. 7. Sedimentation analysis of the effect of various compounds on the binding of [^{3}H]oestradiol-17β by mammary tumour nuclei. Samples (1 g) of tumour tissue were minced and incubated (15 min at 30°C) in Eagle's basal medium (10 ml) supplemented with [^{3}H]oestradiol-17β (5 nmole/l) alone (○) or together with oestradiol-17β or diethylstilboestrol (●), 5-androstene-3β, 17β-diol (△), 5α-androstane-3β, 17β-diol (▲), testosterone (---■---) or 5α-dihydrotestosterone (■), (all at 5 μmole/l). After incubation, nuclei were purified, extracted with KCl (0.4 mole/l) and samples of extract analysed on sucrose gradients [10]. *Sedimentation marker (arrow) was bovine serum albumin ($S_{20,w}$ 4.6S). Direction of centrifugation was from left to right.*

ing oestrogenic potency, the compounds most effective in reducing specific binding of [^{3}H]oestradiol-17β were 5-androstene-3β, 17β-diol, 5-androstene-3α,17β-diol and 5α-androstane-3β, 17β-diol. This observation supports the suggested importance of the 3- and 17β-hydroxyl groups [4,5], although the alcoholic hydroxyl confers less affinity than the phenolic hydroxyl [16]. The 3-hydroxyl in the β-position results in higher affinity than when in the α-position, and the change in orientation caused by the change from a 5-ene to a 4-ene also decreased affinity. The presence of the keto groups rather than the hydroxyl group, e.g. testosterone, 5α-dihydrotestosterone, androstendione, dehydroepiandrosterone and epitestosterone, renders compounds relatively inactive in such competitive binding studies.

The comparative efficiency of the 5-androstenediols and 4-androstenediol is worthy of further note. 4-Androstene-3β, 17β-diol was shown to be relatively inactive in competition for the oestrogen receptor of human uterus [17] and human breast cancer [16], while 5-androstene-3β, 17β-diol was very effective in human myometrial and human breast cancer tissue [14], competing within the physiological range of concentration [18]. This may assume greater importance in rat mammary tissue, wherein 5-androstene-3β, 17β-diol is a metabolite of dehydroepiandrosterone [19]. Overall, these studies suggest a similar specificity of the oestrogen receptor of a number of tissues [4, 16–18].

In view of postulated regulatory roles of 5-androstene-3β, 17β-diol [4, 18], it is interesting to note that it behaves similarly to oestrogenic compounds by apparently binding to the same site as oestradiol-17β (Fig. 6). It is not unusual for 5α-dihydrotestosterone to be inactive as regards the oestradiol receptor (Fig. 2b) [20] since a spectrum of androgenic compounds have been shown to exert physiological effects [see ref. 21], among them 5α-androstane-3β, 17-β-diol [22] and 5-androstene-3β, 17β-diol [23]. Together with the data presented in Figs. 1 and 2, these observations suggest that the variety of 'active' androgens might be reflected in diverse binding characteristics, ranging from nominal androgen receptors to nominal oestrogen receptors.

Those substances which diminish cytosol retention of [^{3}H]oestradiol-17β also prevent nuclear accumulation of [^{3}H]steroid (Fig. 7). This is not unexpected and does not preclude androgen-induced translocation of receptor. We have previously shown [24] that pharmacological doses of androgen can translocate receptor to nuclei and that 5-androstene-3β, 17β-diol was most efficient in this respect. This data [24] agrees with concepts that not only those androgens which bind well to the oestrogen receptor promote its translocation, but also those which bind poorly may effect translocation if the intracellular concentration is sufficiently high [5, 25].

The role of androgens in oestrogen-target tissues remains obscure. Androgen-induced transfer of receptor to nuclei of immature rat uteri has been suggested to provide a pool of receptor inaccessible to oestrogen resulting in depletion of intracellular oestrogen-receptor complex and denigration of synthetic processes [25]. However, high doses of androgen can translocate oestrogen-receptor to nuclei of the same tissue and promote protein synthesis quantitatively and qualitatively similar to that produced by physiological quantities of oestrogen [26]. This would ascribe to androgens uterotrophic and antiuterotrophic responses induced by the same mechanism. However, the involvement of pharmacological doses of an-

drogens may result in aberrations. At physiological concentrations, androgens may deplete binding of oestradiol-17β but may not effect the necessary transformations for nuclear transfer. In this respect, a regulatory mechanism may operate which may be relevant to the study of human breast cancer, especially in view of observations on the competitive properties of 5-androstene-3β, 17β-diol [4, 5 and above].

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Lung Cancer and Postoperative Empyema

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Abstract – *In order to determine the possible effect of postoperative empyema on survival in lung cancer, we studied the registered data for* 148 *male patients operated on between January* 1961 *and June* 1972 *for pulmonary squamous cell and large cell carcinoma.*

In curative cases, the 3- *and* 5- yr *survival rates for the* 14 *patients that had empyema were* 57% *and* 42%, *and for the* 51 *patients without empyema,* 73% *and* 51% *respectively; the survival curves are not statistically different. In reductive cases, the* 3- *and* 5-yr *survival rates for the* 20 *patients with empyema were* 5%, *and for the* 63 *without empyema,* 18% *and* 11%; *the difference in the survival curves is statistically significant* ($P < 0.025$).

It is our conclusion that a postoperative empyema is not a favorable event.

INTRODUCTION

PUBLICATIONS on the effect of empyema on lung cancer survival report different and often misquoted results, but sustain the reputation of a favorable influence [1, 2]. Sensenig, Rossi and Ehrenhaft [3], Leroux [4], Virkkula and Kostiainen [5] are often quoted for offering evidence of the beneficial effect of intracavitary infection following lung resection for tumor. Nevertheless, their reports were not designed to study the empyema effect and no clear conclusion can be drawn.

Among the controlled studies, Cady and Cliffton [6] reported a 5 yr survival rate of 13% for 40 empyema and 35% for the others; Lawton and Keehn [7] reported 21.4% as opposed to 29.8%; Uzzan, Israel-Asselain and Personne [8] reported 25% for both groups.

On the other hand, Takita [9] noted 7 patients suffering from empyema, who survived for 5 yr, i.e. 54% compared to 27% in the control group; Ruckdeschel, Codish, McKneally *et al.* [10] found the 5 yr survival rate for the 18 empyemic patients to be 50% as compared to 18% for the 34 matched control patients.

The interpretation of these results is complicated due to a lack of coherent information on histology, tumor staging, type of operation, presence of postoperative residual neoplastic tissue and statistical methods.

We planned this study to analyze the effect of postoperative empyema on survival among defined patient groups operated for lung cancer, while using the records of the Thoracic Surgery Department of the Hôpital Saint-Pierre, in Brussels.

MATERIAL AND METHODS

The study was carried out on the basis of the recorded cases [11] of patients who underwent lung resection for primary lung neoplasia between January 1961 and June 1972 in the Hôpital Saint Pierre. The criteria of operability and resectability have been defined earlier [12–16].

Using the International T.N.M. Staging System [17], the surgical resections of tumors confined to $T_0 \rightarrow T_2, N_0 \rightarrow N_1, M_0$ (preoperative estimation and postsurgical status) without any postoperative known residual tumor were grouped as curative resections [18]. Palliative or reductive resection was used for the other cases (T_3, N_2). No patient with known metastases was operated upon. Time zero was the day of surgery. Patients who died within 30 days of operation were excluded from both control and empyema groups. All the patients were male smokers. All the empyemas occurred within 6 months of lung resection. In order to obtain homogeneous histological groups, we only studied well differentiated and poorly differentiated squamous cell along with large cell undifferentiated (neither giant cell nor mucinous) carcinomas [19, 20]. Of the 45 recorded cases of empyema (following 349 operations, i.e. an incidence of 12.9%), 36 belonged to this histologic specification.

Another clinical study was conducted at the same time in our service [18, 21] concerning the patients who underwent a curative operation for

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lung cancer: patients received at random postoperative prophylactic complementary radiotherapy. Because of this randomized study and the contraindication of radiotherapy when intracavitary infection occurs, no patient in the curative empyemic group was irradiated. So we only compared the cases without any adjunctive postoperative therapy when the surgery was curative. Fourteen cases of empyema were compared to 51 cases without empyema.

After reductive surgery, the patients were proposed for complementary therapy (radio- or chemotherapy). Since no standard postoperative therapy was planned, the groups with or without empyema had not been selected for postoperative treatment. Twenty cases of empyema were compared to 63 control cases.

Thus the patients studied here were selected according to the 4 following criteria: (1) male smokers, (2) squamous cell carcinoma, (3) survival longer than 1 month, (4) no radiotherapy after curative resections. This left us with a series of 148 patients out of the original 349.

The survival curves were calculated by the Kaplan–Meier method and compared by the adapted Wilcoxon test [22]. We have computed the expected life time (E.L.T.) under exponential hypothesis for each group of patients, defined as the ratio of total observed survival days over number of deaths.

RESULTS

Tables 1 and 2 give the contingency characteristics of the studied groups of patients. Empyema is not related to curative or reductive resection (Table 1, $P>0.5$ on chi-square test). Empyema is not a cause of increased postoperative mortality, as there were only two deaths within a month after the 36 operations complicated by empyema. The occurrence of an empyema is strongly related to pneumonectomy (Table 1, $P<0.001$ on chi-square test). It would be an important statistical parameter, but according to our data, the type of operation does not seem to influence the survival. In this study, the expected life times under exponential hypothesis (E.L.T.) for curative pneumonectomies vs curative lobectomies are 3105 days (9 deaths) vs 3192 days (14 deaths); for reductive pneumonectomies vs reductive lobectomies, the E.L.T.'s are 577 days (16 deaths) vs 813 days (29 deaths). The differences are not significant.

The data shown in Table 1 indicate a poorer prognosis for the patients suffering from empyema (survival status, $P<0.001$ on chi-square test). Empyema does not reduce the recurrence of cancer after lung surgery in either operative group ($P>0.3$ on chi-square test).

The patients with postoperative empyema tend to be older, 59.4 ± 7.3 yr vs 57.5 ± 6.4 yr but the difference is not significant ($P>0.1$ on chi-square test and $P=0.17$ on t-test).

Table 2 analyzes the patients who had reductive operations. Age, assumed postoperative residual tumor and chemotherapy rate are not significantly different. Only the postoperative radiotherapy rates are statistically different (30% vs 52%, $P<0.02$ on chi-square test).

Survival rates (Tables 3 and 4)

Survival rates compared by the appropriate Wilcoxon test are given in Table 3. Each empyema group has a shortened course, which is not due to higher postoperative mortality or to more frequent recurrence (Table 1). Only the survival curve of the patients suffering from empyema after palliative surgery is statistically lower than that of the control group ($P<0.025$). Half-life time and expected life time under exponential hypothesis (E.L.T.) are presented in Table 4. The values of E.L.T. are roughly reduced by half in each empyemic group.

In Table 2, we have noted a significant difference ($P<0.02$) in the postoperative radiotherapy rate between the palliative groups with and without empyema. This potential bias has been further analyzed. In the control group, eleven patients (28%) were surviving at the last follow-up period after postoperative radiotherapy. Their E.L.T. was 604 days. Without radiotherapy, seven patients survived (29%) and the E.L.T. was 753 days. The differences are not significant. Postoperative irradiation and simultaneous occurrence of an empyema tend to decrease the E.L.T.: 217 days vs 426 days (not significant on two-sided F-test).

Due to small populations, we were unable to test the influence of the number and types of causative organisms or the therapeutic methods of managing the empyema. The Karnofsky's index was introduced too late into the study to be assessed.

DISCUSSION

Table 5 recapitulates the reported cases and Table 6 the original controlled studies. For classification and comparison, we tried to use the same terminology. The characteristics are not at all uniform and the data are contradictory. No precise analysis was possible from the reported cases. Even more, the late-occurring empyemas are often included in the series to analyze the

Table 1. *Distribution of patients according to surgical procedure, follow-up and occurrence of postoperative empyema (percent in parenthesis)*

	Operation*			Follow-up					
				Survival status†		Recurrence			
	Aim	Lobectomy	Pneumo-nectomy	Alive	Dead	Yes	No	Not known	Total No.
Empyema	Curative	3 (21.4)	11 (78.6)	2 (14.3)	12 (85.7)	3 (21.4)	5 (35.7)	6 (42.9)	14
	Reductive	1 (5.0)	19 (95.0)	0 (0.0)	20 (100)	10 (50.0)	0 (0.0)	10 (50.0)	20
Control	Curative	32 (62.7)	19 (37.3)	28 (54.9)	23 (45.1)	14 (27.4)	23 (45.1)	14 (27.5)	51
	Reductive	25 (39.7)	38 (60.3)	18 (28.6)	45 (71.4)	34 (53.9)	4 (6.3)	25 (39.7)	63

*Chi-square empyema/type of operation $P<0.001$ (1 d.f.).
†Chi-square empyema/survival status $P<0.001$ (1 d.f.).

Table 2. *Reductive cases: distribution of the patients according to age, presence of residual neoplastic tissue, postoperative therapy and occurrence of empyema*

	No.	Mean age in yr	Residual neo. tissue	Radio-therapy	Chemo-therapy
Empyema	20	57.6 sd 8.3	14	6‡	2
Control	63	58.2 sd 7.3	38	39‡	6

‡Chi-square $P<0.02$.

Table 3. *Survival function in percent by year, excluding the premature postoperative deaths (Kaplan–Meier method)*

		1 yr	2 yr	3 yr	4 yr	5 yr	6 yr	Stat. test Wilcoxon
	Empyema	50	32	26	23	20	4	2.559
	Control	71	55	45	35	30	24	($P<0.02$)
Curative	Empyema	78	57	57	50	42	11	1.502
	Control	90	78	73	61	51	44	N.S.§
Reductive	Empyema	30	15	5	5	5	0	2.285
	Control	56	34	18	11	11	0	($P<0.025$)

§N.S. = not significant.

Table 4. *Survival parameters: the median survival time and the expected life time (E.L.T.) under exponential hypothesis (expressed in days)*

	Curative		Reductive	
	Control	Empyema	Control	Empyema
Median	1791	1398	443	199
E.L.T.	3123	1545	660	363

Table 5. Literature: summary of the reported cases

Authors	Characteristics	No.	Deaths (†)	Alive (follow-up time)	5 yr survivors	Lost
Sensenig [3] 1963	Early empyemas	5	2	1 (33 mths)	1	1
	Late empyemas 9 mths	1	1			
	*18 mths	1			1	
	7 yr	1			1	
in Cady and Cliffton [6] 1967	Without bronchial fistula	34			14	
Leroux [4] 1965	Preoperative empyemas	8	2	5 (2–8 yr)		1
	Postoperative empyemas			all squ. C.		
	Post lobectomy	3	2 (2)	1 (3 yr)		
	Post pneumonectomy	21	11 (6)	6 (1.6–6 yr)	4 (> 10 yr) Squ. C.	
Virkkula, Kostiainen [5] 1970	Curative pneumonectomies for spinocellular cancer					
	Early empyemas	10	3	7 (11–54 mths)		
	Late empyemas					
	*3 yr	1			1	
	4 yr	1			1	
Slack in Moore [1] 1970		24	(6)		2	
Triboulet, Bourcereau [23] 1970	All tumors					
	Postoperative empyemas	29	12 (4)	10 (1–5 yr)	7	

*Date of occurrence of empyema.
†Between parentheses, the number of deaths within a month.
Squ. C.: squamous cell carcinoma.

Table 6. Literature: summary of the controlled studies (using our own classification)

	Lawton [7] Keehn 1972		Takita [9] 1970		Cady, Cliffton [6] 1967				Ruckdeschel [10] *et al.* 1972				Uzzan [8] *et al.* 1974	
†	E+	E−	CE+	CE−	E+	E−	CE+	RE+	E+	E−	CE+	CE−	E+	E−
Histology *squ. C.			79%		72%		72%	68%	71%	65%			100%	100%
*ana. C.			14%		2%		—	4%	23%	20%				
*adC.			7%		15%		11%	20%	6%	15%				
*bronch. C			—		11%		17%	8%	—	—				
Pneumonectomy	34	933	9	93	35	217	18	25	15	21	11	22	16	122
Lobectomy			5	85	5	116			3	13			—	—
1–36 months survival rate %	32.1	42.2					31.2‡	8.0‡	60	37	90	54		
1–60 months survival rate %	21.4	29.8	53.8‖	27.2	12.5	34.5	31.2‡	0	50	18	73§	23§	25.0	25.4
Statistical significance	N.S.				$P<0.01$				$P=0.007$		$P=0.008$		N.S.	

*Squ. C.: squamous cell carcinoma.
Ana C.: anaplastic cell carcinoma.
AdC.: adenocarcinoma.
Bronch. C.: bronchoalveolar carcinoma.
†E+: Empyema, E−: control, C: curative cases, R: reductive cases.
‡The survivors had squamous cell carcinoma.
§Histology for the 5 year survivors is in CE+: 86% squ. C., 14% ana.C.; in CE−: 83% squ. C., 17% adC.
‖Histology for the 5 yr survivors is: 6/7 squ. C. 1/7 oat cell C. One patient developed an empyema 2 yr after surgery.

survival rates. In the controlled studies, there is insufficient information to compare the observed differences. The study of Ruckdeschel *et al.* [10] with the selection of matched control patients has been criticized by some authors [1, 8] because of the low survival rate in the control group, the small samples and some discrepancies in the survival patterns. These studies and ours showed that the occurrence of empyema is related to pneumonectomy.

The misquotations of some reports are surprising. From Leroux [4], Cady and Cliffton [6] and Takita [9] report the 5-yr-survival rate to be 33% for 15 pneumonectomies, Uzzan *et al.* [8] report a 10/21 survival rate, while Ruckdeschel *et al.* [10] report the 10-yr survival to be 4 out of 6 patients. From Sensenig *et al.* [3], Cady and Cliffton [6] and Takita [9] report 4 five-yr survivors out of 7 pneumonectomies, Ruckdeschel *et al.* [10], 3 five-yr survivors and Uzzan *et al.* [8], 5 five-yr survivors. From Virkkula and Kostiainen [5] who report the treatment of 10 patients with empyema without analyzing the "cancer" survival rate, Ruckdeschel *et al.* [10] and Uzzan *et al.* [8] report a 4/7 rate after 5 yr. From Cady and Cliffton [6], Takita [9] and Uzzan *et al.* [8] report 5 five-yr survivors, the first out of 24 patients and the latter out of 35 patients.

Sensenig *et al.* [3] were the first to suspect a beneficial effect of empyema on lung cancer survival, possibly indicating stimulation of host defence against residual tumor cells. Many mechanisms may be advocated: physical blockage of lymphatics by the inflammatory process [3], tumoricidal effect of bacterial toxins [1, 3, 10] or of fever due to infection [24] (but Cady and Cliffton did not find a favorable effect of fever), or increased antigenicity of tumor cells by bacterial hydrolytic enzymes such as neuraminidase [25]. Cady and Cliffton [6] reported a trend (statistically not significant) for 5 yr survivors to have more infections due to staphylococcus and streptococcus pyogenes, to have a greater number of bacterial species per infection and to have fewer infections with only one species of organism. Ruckdeschel *et al.* [10] postulated an immunological hypothesis of a non-specific tumoricidal effect by lymphokines released by lymphocytes activated by bacterial antigens.

This study was primarily planned to examine the attractive hypothesis that empyema could enhance host defences.

It is a retrospective study and therefore cannot eliminate chronologic and detection biases. There are three further possible biases, age distribution, operative procedures (Table 1) and radiotherapy in palliative cases (Table 2). Age is in itself a cause of increased lethality [25]. The influence of age on the observed survival rates was minimized by the results of statistical tests concerning age distribution between the empyema and the control groups, which never reached statistical significance. The operation parameter (more frequent pneumonectomies in empyema groups could be disregarded according to E.L.T. values comparable in operative subgroups. The influence of radiotherapy in palliative cases with postoperative empyema was further analyzed. Irradiation and empyema together tend to lessen the E.L.T.: 7 months vs 14 months but the difference is not significant. Radiotherapy had no influence on survival in the control group.

In palliative cases, survival curves are significantly lower for patients suffering from empyema ($P < 0.025$). In curative cases, empyemic patients have a shorter survival. E.L.T.'s are of 51 months vs 104 months, but the survival curves cannot be differentiated on a statistical basis.

Therefore, as far as this study is concerned, by following up patients with spinocellular and large cell undifferentiated bronchogenic carcinoma, without postoperative prophylactic irradiation in curative cases, we were unable to demonstrate a favorable effect of empyema. We found no beneficial effect of empyema on lung cancer survival (Tables 3 and 4) or recurrence rates (Table 1). Furthermore, postoperative empyema contraindicates prophylactic radiotherapy which has been previously proved to increase survival rate in curative cases [27]. However, because the observed decrease of survival rates in cases with empyema may be due to the three possible biases discussed above, we cannot be certain that the unfavorable effects observed are due to empyema.

The literature is contradictory (Tables 5 and 6) and we think it will remain so. Empyema is a rare event (5–15%) and, therefore, authors' report only small numbers of patients. Unfortunately, the survival rate in lung cancer depends on many variables. Some are easily controllable i.e. age, sex, race, smoking habits and occupational hazards. For a comparative study, we need full information about histology and staging of the tumor. However, in retrospective studies, there are uncontrolled variables: methods of detection of metastases have changed and improved e.g.: percutaneous lung biopsy and mediastinoscopy vs Daniel's biopsy, osseous scintigraphy vs radiologic findings, hepatic scintigraphy and laparoscopy vs biologic data and laparotomy, cerebral scintigraphy and the new computerized transverse axial tomography vs neurological examination

and E.E.G. and so on. Occurrence of empyema following a bronchial fistula probably has a worse prognosis, as mentioned by Sensenig *et al.* [6]. The date of occurrence and duration of empyema seems likely to be crucial for its presumed action on host defences, as cell-mediated immunity to tumor-associated antigens has been shown to be depressed for 6–22 days after surgical operation [28]. Also, adjuvant chemotherapy and radiotherapy are known to decrease host defences against infectious diseases.

For all these reasons, we believe that a strictly conclusive clinical study is practically impossible and incidental empyema will remain a subject of controversy. A partial answer would be possible by studying the immunological status of each patient, while applying the new immunological methods: lymphocytic inhibition of macrophage migration by tuberculin, encephalitogenic protein and tumor extract, lymphocyte transformation by PHA, skin test with cell-mediated immunogens [29], rosette formation and their inhibition by the antilymphocyte globulin [30], and skin tests with tumor extract [31]. The immunological status of patients suffering from empyema has never been assessed. The ultimate answer is in clinical studies of experimental empyema with B.C.G. [32, 33] or with *Corynebacterium parvum* in randomized patients.

Despite our retrospective study and our review of the literature, we were unable to demonstrate a favorable effect of empyema on lung cancer survival.

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The Effect of Subcutaneous Administration of Oestrogens on Plasma Oestrogen Levels and Tumour Incidence in Female Rats*

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Abstract—*A study on a possible synergistic effect of oestrogens and ionizing radiation on mammary carcinogenesis required the use of female rats with elevated plasma levels of oestrogens in a longitudinal experiment. Following subcutaneous implantation of oestrogen pellets, plasma oestrogen levels were monitored as a function of time after administration, procedure of pellet preparation, chemical nature of the oestrogen and concentration of the oestrogen in the pellets. 17β-oestradiol (E_2), 17β-oestradiol 3-benzoate (E_2B) and 17α-ethynyloestradiol (EE_2) were studied in this respect. In initial studies, 5–20 mg E_2B were implanted; this resulted in extremely high peripheral plasma levels and caused deterioration in the physical condition of the animals. After a latent period of 10–12 months, pituitary and mammary tumours were observed in a considerable number of animals.*

Experiments with pellets containing tritium labelled 17β-oestradiol were performed in order to compare the kinetics of hormone release from pellets produced by various techniques. Based on the results of these experiments, implantation of oestrogen in a paraffin-cholesterol pellet was adopted for all further experiments. Peripheral plasma levels of oestrogen are reported for 17β-oestradiol and 17α-ethynyloestradiol at various time intervals after administration of pellets containing 1.0 and 2.5 mg of oestrogen to intact and ovariectomized rats.

INTRODUCTION

IN VIEW of the relatively high incidence of mammary cancer in the human, it is of great importance to unravel the mechanism of mammary gland carcinogenesis in experimental models. Previous studies have suggested a relationship between the occurrence of mammary tumours in the human and several factors, including radiation [1], nutritional status [2] and oestrogen use [3, 4]. Furthermore, it has been shown [5, 6] that, in the rat, oestrogen (diethylstilboestrol, DES) and ionizing radiation (X-rays and neutrons) act synergistically to produce mammary tumours. In these studies, DES was administered to female A × C rats via subcutaneous implantation of hand pressed DES-cholesterol pellets containing 5 mg DES and 15 mg cholesterol. However, the authors provided no data on the actual levels of DES in peripheral plasma after implantation. For a proper evaluation of the possible carcinogenic effect of the administered oestrogen, it is important to know the relationship of the oestrogen plasma level after oestrogen administration to the physiological oestrogen level.

The present study was designed to define the hormonal status of experimental animals during combined oestrogen and radiation treatment in terms of oestrogen levels in peripheral plasma, rather than in terms of the amount of oestrogen implanted in the animal. In this respect we have studied 17β-oestradiol (E_2), 17β-oestradiol-3-benzoate (E_2B) and 17α-ethynyloestradiol

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(EE_2), since these are either naturally occurring, therapeutic or contraceptive oestrogens.

MATERIAL AND METHODS

Animals

Female Wistar WAG/Rij rats either intact or after hystero-ovariectomy (Ovex) were used throughout this study. The animals were kept in rooms with controlled temperature, light and humidity conditions and received tap water and standard dry pellets *ad libitum*. Oestrogen pellets were implanted subcutaneously in the dorsal region of the neck under light ether anaesthesia. Blood was taken from the tail under light ether anaesthesia at regular intervals after pellet implantation. The blood was collected in heparinized tubes and centrifuged within 2 hr to obtain plasma, this was stored at −20°C until assay.

Preparation of oestrogen pellets

Oestrogen pellets were prepared by one of the following methods:

Method A

The oestrogen was mixed with an appropriate amount of cholesterol. The mixture was pressed with a small hand-operated press to give pellets weighing 21.8 ± 2.3 mg (mean ± S.D., $n = 109$) with a cross section of approximately 4 mm.

Method B

The oestrogen was mixed with cholesterol. The mixture was melted in an oil bath at 190°C and was aspirated into silicon tubing of 3 mm cross section in 0.5 ml portions. After cooling the tube at ambient temperature, segments of 2–3 mm were cut.

Method C

A mixture of oestrogen, cholesterol and paraffin was made and pellets were prepared as described for method B. Cholesterol and paraffin were used in a 1 : 10 (w/w) ratio [7]. The average pellet weight was 20.3 ± 1.3 mg (S.D., $n = 100$).

Pellets selected for implantation weighed 19–21 mg. Tritiated oestrogens were included in the pellets in some experiments and steroid release was monitored by liquid scintillation counting of small blood samples taken at regular intervals after pellet implantation. In a counting vial, 0.2 ml of heparinized blood, 0.2 ml of isopropyl alcohol and 0.2 ml of a 30% solution of hydrogen peroxide were mixed and incubated at 60°C for 1 hr to decolorize the blood. The residue was dissolved after incubation with 2 ml of Soluene (Packard) for another 2 hr at 60°C. Finally, 15 ml of Permablend scintillation cocktail (Packard) were added and the radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation counter with a counting efficiency of approximately 10%.

Steroids

17β-Oestradiol and 17α-ethynyloestradiol for implantation were gifts from Organon, Oss, The Netherlands. 17β-Oestradiol 3-benzoate was purchased from Sigma, St. Louis, U.S.A. 17β-Oestradiol and 17α-ethynyloestradiol for use as standards in radioimmunoassay were obtained from Steraloids, Pawling, New York, U.S.A. (2,4,6,7,-^{3}H)-17β-Oestradiol was obtained from the Radiochemical Centre, Amersham, United Kingdom and had a specific activity of 85 Ci/mmole. (6,7-^{3}H)-17α-Ethynyloestradiol with a specific activity of 40 Ci/mmole was purchased from New England Nuclear, Frankfurt am Main, Germany. Radioactive steroids were purified by paper chromatography using a system light petroleum:benzene:ethanol:water, in the ratio of 5:5:7:3 by volume. Purity of radioactive steroids was checked every two months.

Steroid estimations

Plasma 17β-oestradiol was assayed as previously described by De Jong, Hey and van der Molen [8]. Cross reaction of 17β-oestradiol 3-benzoate in the 17β-oestradiol radioimmunoassay was 5%. Plasma 17α-ethynyloestradiol was measured essentially as described by Warren and Fotherby [9], but the method was adapted to the protocol for oestradiol radioimmunoassay.

E_2 and EE_2 were not separated by the chromatographic procedure used. We do not expect, however, that endogenous 17β-oestradiol interfered with the assay of EE_2 in the EE_2 treated rats, because the cross-reaction of E_2 with the EE_2 antiserum was 3.5% and because it appears reasonable to assume that only very little E_2 was present after administration of EE_2 which will suppress pituitary lutropin secretion and ovarian E_2 secretion.

Scoring of tumours

Animals which developed mammary tumours were killed either when tumour size was 5–6 cm, or as soon as indicated by the physical condition of the animals. Autopsy was performed on all animals and the occurrence of tumours was registered. Pituitary tumours were classified as such after histological examination.

RESULTS

In an initial experiment E_2B-cholesterol pellets were prepared according to method A.

These pellets contained 5 mg E_2B and 15 mg cholesterol. From 1 to 4 pellets were implanted in intact and ovariectomized rats. Blood samples were taken at day 0, 3, 7, 14 and 30 days after implantation and E_2 was assayed in the plasma. The results for the intact animals are given in Table 1. The results obtained for the ovariectomized animals were essentially the same. The condition of all animals was affected by the oestrogen administration and loss of hair and a decrease in body weight were generally observed. Furthermore within a latent period of 10–12 months, almost all rats (92%) developed pituitary tumours; some of the intact animals also developed mammary tumours. The tumour incidence in this group of animals is shown in Table 2; control animals were still tumour free. In view of the high peripheral plasma E_2 levels measured after administration of E_2B, which reflect a pharmacological rather than a physiological condition, it was decided to decrease the amount of oestrogen to be administered to the animals. Consequently, the second experiment was designed to compare three different methods of pellet preparation. Two doses of E_2 which were lower than the amounts of E_2B used in the first experiment were used. The results obtained are presented in Fig. 1 and show that implantation of the pressed E_2-cholesterol pellets (method A) tends to give higher plasma levels than the other two methods, especially during the first 40 days after implantation of the pellets. This observation was confirmed by the results of the measurements of radioactivity in the blood after implantation of pellets containing tritiated E_2; these are summarized in Table 3. The half-life of radioactivity in the blood was calculated from disappearance curves of plasma radioactivity. The part of the curve after the onset of the decrease in plasma E_2, which normally occurred about 4 days after implantation, was used for this calculation.

In a third experiment, a comparison was made of the oestrogen release from pellets prepared by method C for implantation of E_2 and EE_2 in intact and ovariectomized rats. The results of this experiment are shown in Fig. 2. There appears to be no difference between the rate of release of E_2 and EE_2 in intact and ovariectomized rats.

DISCUSSION

The present results show that, in the rat, an elevated level of plasma oestrogen can be effectuated and maintained by the subcutaneous

Table 1. *Plasma* 17β-*oestradiol in intact female rats after implantation of pellets containing* 5 mg 17β-*oestradiol* 3-*benzoate and* 15 mg *cholesterol. Results are given as means* ± *S.E.M.* (*n*). *For n* = 2 *individual results are given. All results are expressed as* pg/ml

Time after implantation (days)	17β-oestradiol (pg/ml) number of pellets implanted 0	1	2	3	4
0	56 ± 12 (5)				
3	29 ± 3 (3)	650 – 1000	1160 – 2250	1438 ± 238 (3)	3720 – 2080
7	44 – 69	389 ± 74 (3)	882 ± 32 (3)	973 ± 90 (3)	1162 ± 190 (3)
14	103 ± 14 (5)	484 ± 38 (4)	639 ± 63 (5)	1088 ± 137 (5)	1292 ± 133 (5)
30	71 ± 9 (6)	285 ± 18 (4)	404 ± 48 (5)	841 ± 97 (5)	1032 ± 190 (6)

Table 2. *Tumour incidence in intact and ovariectomized rats after implantation of* 17β-*oestradiol* 3-*benzoate* (E_2B) *pellets*

Number of pellets implanted	Total amount of E_2B implanted (mg)	Total number of animals	Latent period (months)	Number of pituitary tumours	Rats with mammary tumours
1	5	intact 5	10	5	4*
		ovex 4	10	4	—
2	10	intact 5	10	5	2†
		ovex 4	12	3	—
3	15	intact 4	11	4	—
		ovex 5	10–11	4	—
4	20	intact 5	8–10	4	1†
		ovex 5	10	5	—

*3 Multiple, 1 single tumour observed.
†Single tumours observed only.

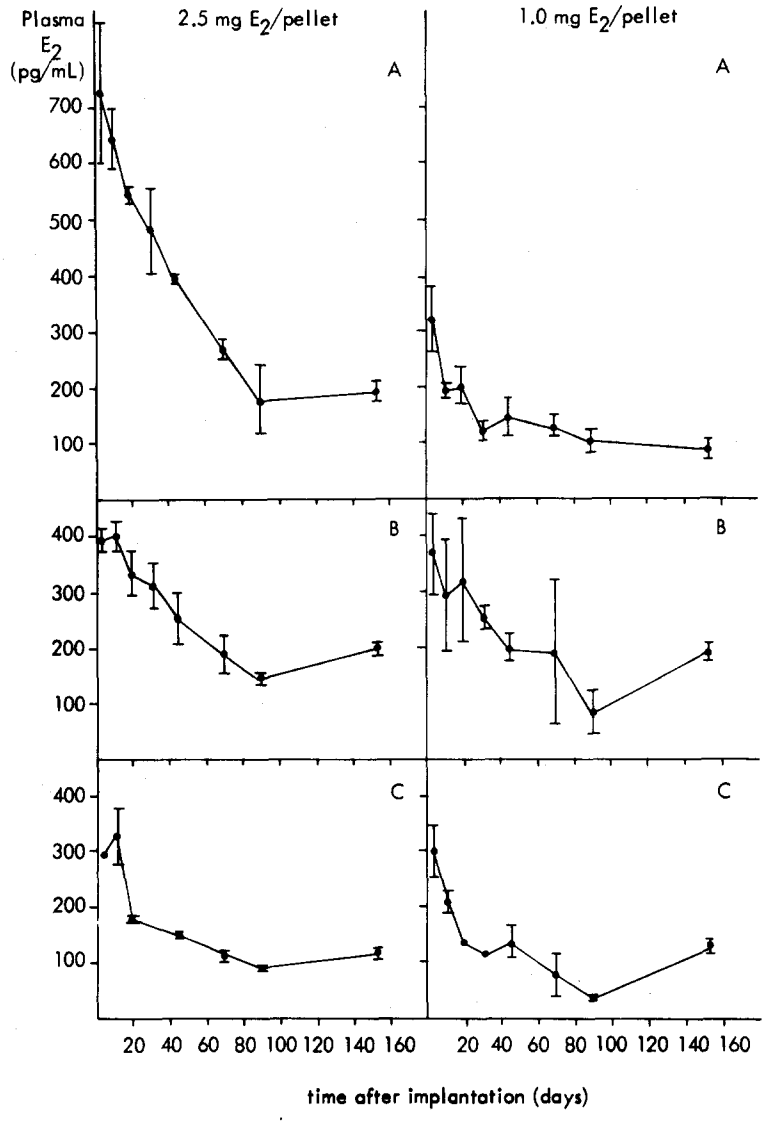

Fig. 1. Plasma 17β-oestradiol (E_2) levels at different times after implantation of an E_2 pellet, prepared by three different methods (see Material and Methods) and containing different amount of E_2. Results are given as means ± S.E.M. ($n \geqq 3$), means and range ($n = 2$) or as individual values.

Table 3. Rate of disappearance of tritiated 17β-oestradiol from the blood of female rats after implantation of pellets containing 1 mg of 17β-oestradiol, prepared by different methods

Pellet preparation method	$t_{1/2}$ (days)
A	7,8
B	9,5
C	10,9

Half-lives ($t_{1/2}$) were calculated as the time in which radioactivity in the blood decreased by a factor 2, starting at the top of the disappearance curves.

(s.c.) implantation of an oestrogen-containing pellet in the dorsal region of the neck. The initial plasma oestrogen levels after implantation depend on the method by which the pellets were prepared. From the differences in plasma oestradiol levels immediately after implantation (Fig. 1) and the shorter half-lives of radioactivity in the blood (Table 3) it appears that the release of oestrogen from pellets prepared by pressing an E_2-cholesterol mixture is more rapid than from pellets prepared by melting an E_2-cholesterol–paraffin mixture. There was no apparent difference between the rates of release of E_2 and EE_2 from cholesterol–paraffin fused pellets, nor did ovariectomy affect the plasma level of these oestrogens (Fig. 2). Other applications of sustained release hormonal preparations have been described by Kincl *et al.* [10]; Sundaram and Kincl [11]; Reddy and Prasad [12] and Coutinho *et al.* [13], who used a dimethylpolysiloxane pouch (silastic implant) through which the steroids have to diffuse before they can enter the circulation. To our knowledge, no results have

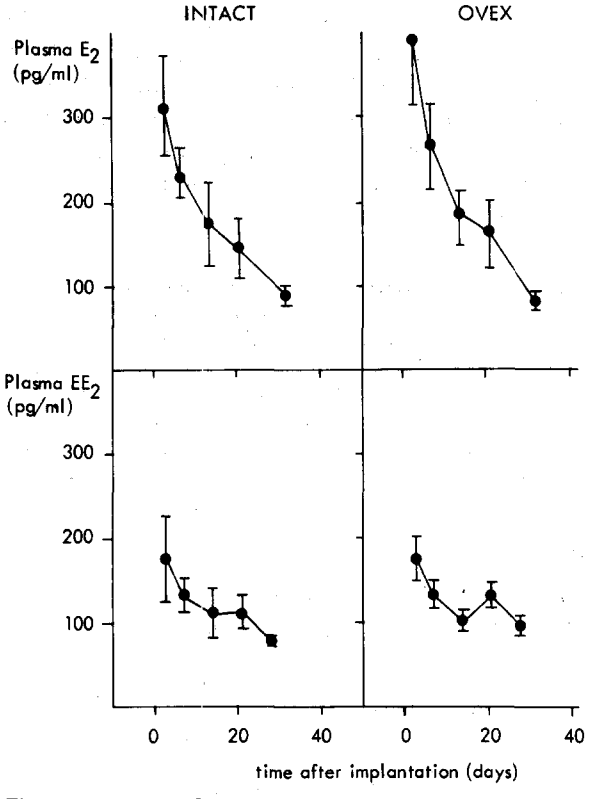

Fig. 2. Plasma 17β-oestradiol (E_2) and 17α-ethynyloestradiol (EE_2) in intact and ovariectomized (OVEX) rats at different times after implantation of an oestrogen–cholesterol–paraffin-pellet containing 1 mg of oestrogen (method C). Results are given as means ±S.E.M. ($n \geqq 3$).

been published on the plasma levels of oestrogens achieved after implantation of such silastic pouches.

From the results in Figs. 1 and 2, it is clear that the plasma oestrogen level reached after implantation is not very constant. In most of the experiments, plasma oestrogen levels were highest during the first month after implantation and gradually decreased thereafter. This might be due to an increase in the oestrogen metabolizing capacity of the liver due to continuous exposure to high levels of oestrogen. On the other hand, it was observed that pellets which were removed from the animal were encapsulated in fibrous tissue, which could have interfered with the transfer of oestrogen from the pellet into the circulation. Pellets prepared by method C which had been implanted for more than 150 days were found to contain still more than half the amount of E_2 originally present (results not shown). The results of our studies with different amounts of E_2 administered in a single pellet (Fig. 1) might reflect that the release of oestrogens was dependent on the surface of the pellet. A dose–response relationship was not observed in this experiment; however, it did occur in the first experiment with E_2B (Table 1), where different amounts of oestrogen were administered by varying the number of the pellets, i.e. a larger amount of oestrogen was administered via a larger surface.

In studying a possible (co)carcinogenic effect of oestrogens by using a synthetic steroid, like EE_2, it is important to consider the possibility that the metabolism of the synthetic steroid may differ from that of natural oestrogens. In order to obtain the results presented in Fig. 2, both E_2 and EE_2 were administered as oestrogen–cholesterol–paraffin pellets containing 1 mg of oestrogen. Yet the E_2 plasma levels rose higher than the EE_2 plasma levels, suggesting a higher metabolism of EE_2. In the human, there appears to be a close similarity between the metabolism of EE_2 and natural oestrogens, at least qualitatively [14]. EE_2 appears to be metabolized slower than E_2. In addition it has been shown [15] that after administration of tritiated EE_2 to women, 8–10% of the radioactivity in the urine could be identified as "de-ethynylated". This de-ethynylation was not observed in incubation studies with rat liver slices [16]. These possible differences between the metabolism of E_2 and EE_2 and the scarcity of data on the metabolism of EE_2 in the rat should be considered when the effects of EE_2 and E_2 are compared.

The occurrence of pituitary tumours in rats following administration of high amounts of oestrogens (Table 2) confirms similar observations of Shellabarger *et al.* [6]. The development of these tumours might have been due to a superstimulation of the prolactin secreting cells in the pituitary. In turn, the prolactin secreted by the hyperplastic pituitary could have caused the initiation of mammary carcinogenesis, since it has been emphasized [17, 18] that prolactin is the main agent in hormonally induced mammary carcinogenesis, whereas oestrogens would act mainly by promoting prolactin secretion. On the other hand, high amounts of oestrogens decrease prolactin binding activity and tumour growth in experimentally (7, 12 dimethylbenz(A)anthracene) induced mammary tumours in the rat [19]. These literature data seem to contradict each other, probably because the exact mechanism of mammary carcinogenesis is as yet ill understood. It has been shown by van der Gugten, Boot, Röpcke and Kwa [20] that a higher dose of oestrogens is required to initiate an elevated prolactin secretion in rats than in mice. Once established, however, a relatively low dose of oestrogen maintains prolactin secretion at a higher level. From the results in Table 1, it can be concluded that the latter situation might prevail in the rats treated with E_2B pellets. In view of these

considerations, it is quite possible that the carcinogenic effect of oestrogens on the mammary gland reported by Segaloff and Maxfield [5]; Shellaberger *et al.* [6] and found in the present study, is an oestrogen mediated prolactin effect rather than a direct effect.

The occurrence of mammary tumours in intact rats and the absence of such tumours in ovariectomized rats in spite of oestrogen administration could suggest the existence of another ovarian factor which is involved in the induction of mammary tumours. In mice and rats a synergistic action of progesterone and oestrone on prolactin-induced mammary tumours has been described [20, 21]. In contrast, others reported that progesterone may protect the mammary gland from becoming neoplastic [22]. Experiments with larger groups of animals are in progress to confirm our observations on the effect of ovariectomy on mammary tumour development and to study the role of prolactin and progesterone in this respect.

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Tissue Culture and Transplantation Studies on Canine Mammary Carcinoma*

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Abstract—*Twenty-three of 27 canine mammary tumours grew in culture following overnight trypsination at room temperature. A solid carcinoma and a tubular adenocarcinoma are probable cell lines. The adenocarcinoma appeared fibroblastic in tissue culture but when transplanted into an immunosuppressed dog produced an adenocarcinoma similar to that in the original dog.*

INTRODUCTION

IN THE bitch malignant mammary tumours account for 25% of all neoplasms. The majority of these are carcinomas and can be subdivided into papillary, tubular, solid and anaplastic types. Other forms such as spindle cell, squamous and mucinous carcinomas may be seen but are rare [1]. Bostock [2] found that invasive carcinomas in the dog have a very poor prognosis, metastasise widely and may provide a good model for breast cancer in women.

The present experiments were designed to provide mammary carcinoma cell cultures for further virological and immunological studies and used tumours similar in morphology to those which have previously been fully described [2].

MATERIAL AND METHODS

Twenty-six primary tumours and one metastatic mammary tumour in the lungs were obtained within a few minutes of mastectomy or euthanasia of the dog. Following dissection of the mass and elimination of necrotic material apparently viable tumour was diced into 1–2 mm pieces and placed in 50 ml of 0.25% trypsin in Hanks BSS at room temperature with stirring. In some cases sufficient cells were obtained in 2 hr and overnight treatment usually resulted in at least 10^8 cells, with a viability between 63 and 90% using the trypan blue exclusion test.

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Medium

The complex medium described by Cailleau *et al.* [3], containing supplements of insulin, cortisol, dexamethasone and glutathione, was used for the majority of primary cultures. Penicillin (200 i.u./ml) and streptomycin (100 μg/ml) were also added since the sterility of surgically excised tumours could not be guaranteed. Some tumours were also grown in TC 199 or RPMI 1640 with the addition of 20% foetal calf serum.

Transplantation into Nude *mice*

Outbred *Nu* mice† were barrier-maintained and bred by crossing male homozygotes *Nu Nu* with female heterozygotes *Nu*+. The offspring were injected with 10^7 or 5×10^7 tumour cells subcutaneously at 6–8 weeks of age [4].

Transplantation into dogs

Neonatal dogs were immunosuppressed with antilymphocyte serum (A.L.S.) as previously described [5] and injected with 8×10^7 epithelial type cells from a solid carcinoma metastatic to lungs. Injections were made at the 18th and 65th passage.

A further neonatal dog was given 25 R whole body X-irradiation on the day of birth and then received A.L.S. three times weekly. In this dog 5×10^8 cells from the fifth passage of a primary tubular adenocarcinoma were injected subcutaneously.

RESULTS

Maintenance in tissue culture

Three out of 27 tumours did not grow and a fourth tumour was heavily overgrown with mould.

Fifteen tumours died out between 2–17 passages and 8 tumours are still alive between 3 and 105 passages.

A solid carcinoma which originated from metastatic tumour in the lung has been in culture for over 2 yr and has been passaged 105 times. The tumour was originally grown in TC 199 medium with 20% foetal calf serum in several flasks. At 6 days one culture flask showed predominantly fibroblastic type cells and another flask predominantly epithelial-type cells. Other flasks were of mixed type. Following the 5th passage at 4 months, rapid growth of the epithelial type cells occurred and was subsequently maintained. After the 25th passage (1 yr) the cells were transferred to the complex medium where the growth rate increased further, the cells at this stage having a doubling time of 4 days and a karyotype of 86 chromosomes (the normal dog cell has 78 chromosomes, 76 of which are acrocentric).

The fibroblastic-type cells were also maintained in culture and were transferred to the complex medium at one year. However, by 14 months the growth-rate slowed and the cultures died out at the 39th passage.

A primary tubular adenocarcinoma was trypsinised overnight and the cells were cultured in the complex medium with 15% foetal calf serum. Growth was very rapid and by the 12th day the culture had reached its 5th passage and consisted of fibroblastic-type cells growing in dense plaques (Fig. 1). The tumour has now been passaged 50 times and has maintained its original morphology *in vitro*. On the 36th passage there was a karyotype of 96 chromosomes (Fig. 2).

Transplantation into Nude *mice*

Following the inoculation of 10^8 epithelial type cells of the solid carcinoma into 2 sites in a *Nude* mouse, tumour nodules became palpable after 22 days at which time the animal was killed. One tumour was used for histochemical studies and the other for growth in tissue culture. Histologically the appearance of the transplanted tumour was similar to the original metastatic solid carcinoma. The cells obtained from the transplanted tumour have been maintained in tissue culture and have a similar appearance and doubling size to those of the original tumour cells maintained *in vitro* throughout. The fibroblastic-type cells from the original solid carcinoma after 10 months in tissue culture were injected into a *Nude* mouse but there was no evidence of nodule formation over a 9-month period.

Tissue culture cells from the tubular adenocarcinoma injected at the 6th passage into a *Nude* mouse produced a 1 cm diameter tumour 55 days after injection. The histological appearance was of a tubular adenocarcinoma with a similar morphology to that of the original canine tumour.

Transplantation into dogs

Solid carcinoma. No detectable growth occurred with cells of the 18th passage in one puppy. In two further puppies injected subcutaneously with cells from the 65th passage tumour-like nodules appeared at 3 months but later regressed.

Tumour 2. By 22 days a tumorous mass (approximately 2 × 2 cm) had developed at the site of injection of the tissue culture cells. The histological appearance of this tumour (Fig. 3) was similar to the original tubular adenocarcinoma (Fig. 4) and cells placed in tissue culture had a similar appearance and doubling time to the cells used in the transplantation.

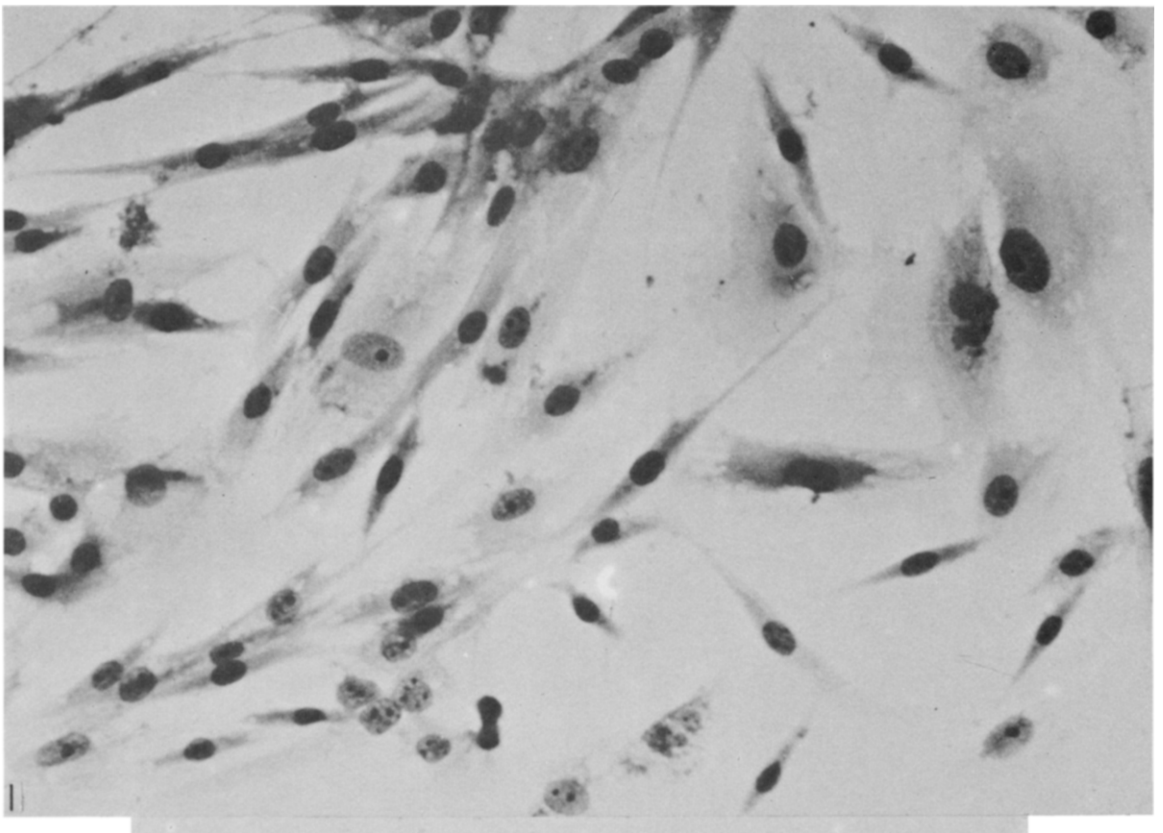
DISCUSSION

There has been a general lack of success in attempts to establish tumour cell lines from solid specimens of human breast carcinoma but better results have been obtained using pleural effusions [3, 6].

Our experience in dogs indicates that rapidly growing or metastatic tumours are the best starting material. Overnight incubation with trypsin at room temperature has consistently yielded better results than trypsinisation for 1 hr at 37°C or at +4°C overnight. Growth of fibroblasts and epithelial cells appears to vary from flask to flask and if a sufficient number of cells is available, seeding into multiple flasks is recommended.

The cells from the tubular adenocarcinoma which has been described appeared more fibroblastic than epithelial in tissue culture yet produced a typical adenocarcinoma when transplanted into an immuno-suppressed dog. It may be that in some human tumours cells of fibroblastic appearance grow in tissue culture and are wrongly discarded as being stromal cells while in fact they are malignant. Transplantation into *Nude* mice may resolve some of these difficulties.

Acknowledgements—We thank our colleagues who are in practice in many different areas for supplying us with the tumour material.



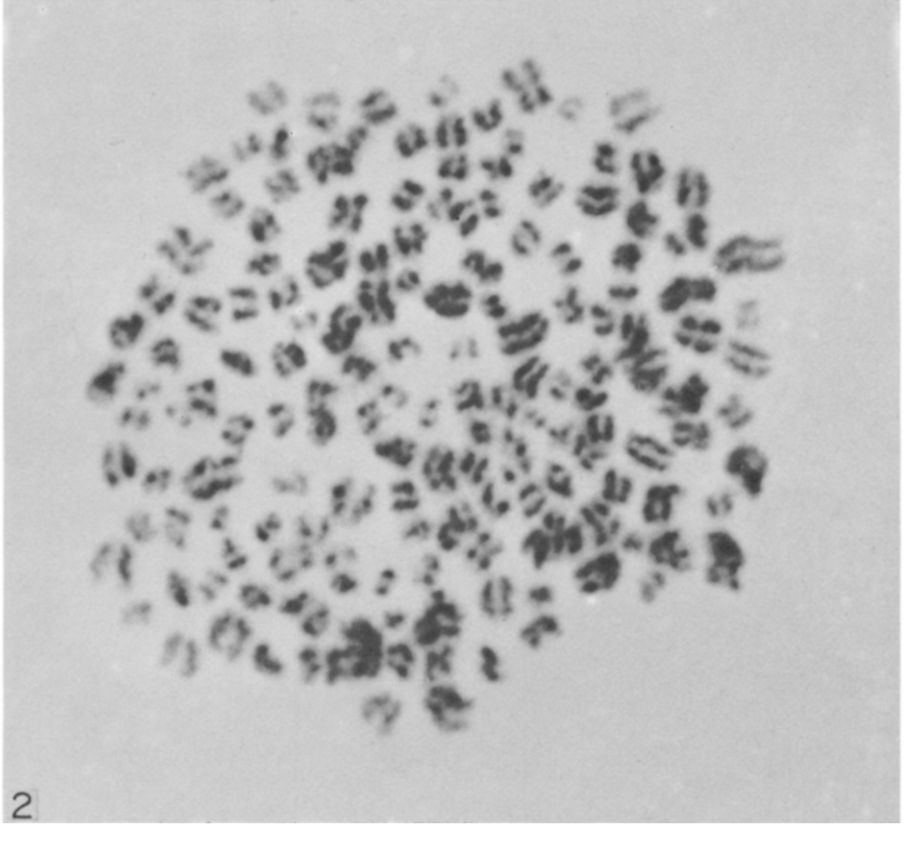


Fig. 1. *Tubular adenocarcinoma in tissue culture with fibroblastic appearance. Fifth passage.*

Fig. 2. *Karyotype of the adenocarcinoma*—96 *chromosomes.*

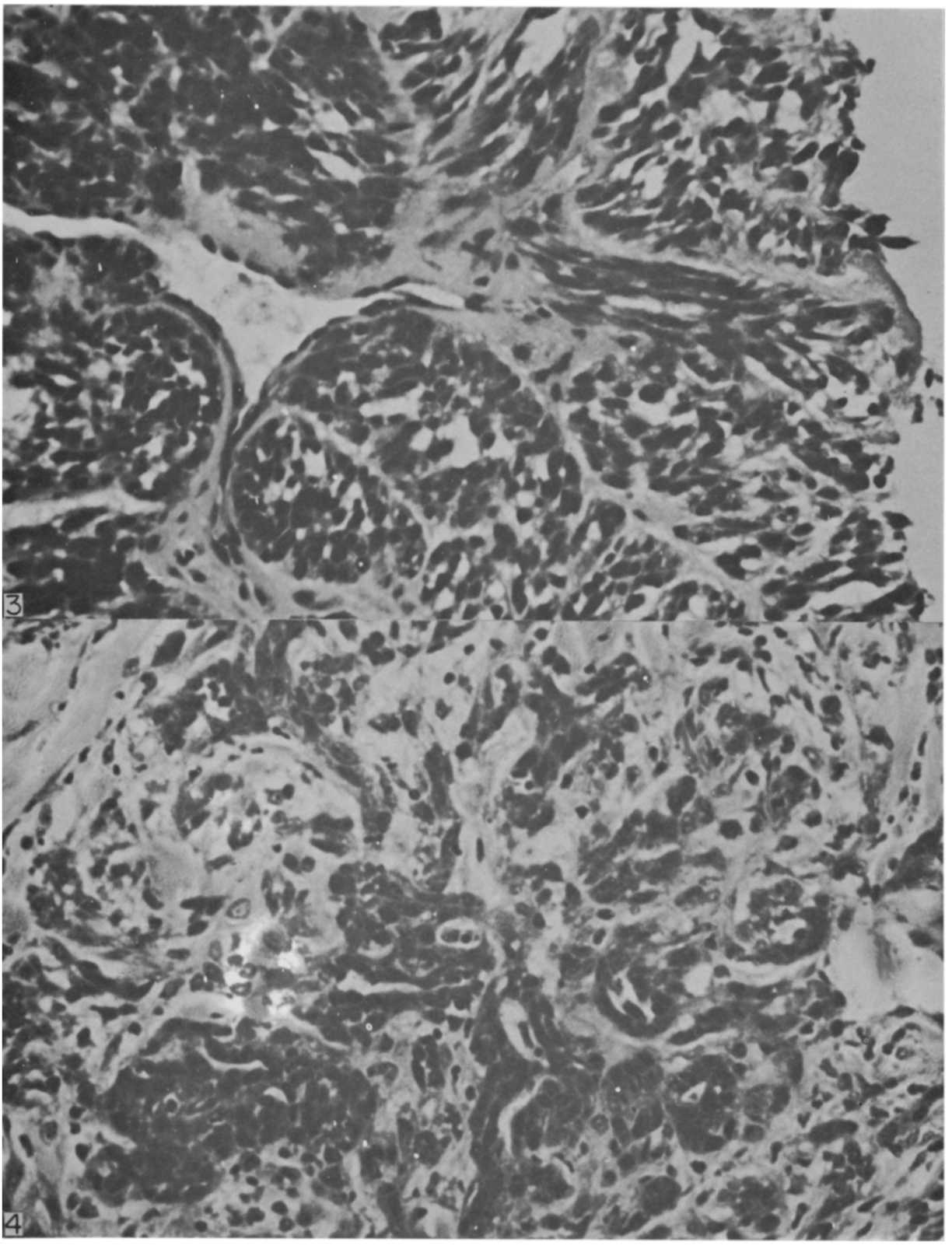


Fig. 3. *Histological appearance of tumour growing in an immunosuppressed dog from fibroblastic-like cells in tissue culture. The tumour is an adenocarcinoma similar to the original tumour. See Fig.* 4.

Fig. 4. *Tumour* 2. *Histological appearance of adenocarcinoma of mammary gland in the donor dog.*

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Letter to the Editor

Effect of the Combination of *Corynebacterium Parvum* and Levamisole on Murine Tumors*

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MUCH interest is currently centered in the use of non-specific immunostimulants in cancer treatment in view of the effectiveness of this approach when employed simply or in association with chemotherapy, in various experimental tumor models and in at least a number of human malignancies [1–3]. Although many studies are now available on the antitumoral activity of compounds such as BCG, *Corynebacterium parvum* (*C. parvum*) or levamisole (Leva), little attention appears so far to have been devoted to explore the therapeutic potential of combinations of immunostimulants. In view of the fact that quantitative as well as qualitative differences appear to exist in the mode of action of this type of compounds [2, 4], it could in principle be expected that appropriate combinations of immunomodulators may result in additive or synergistic effects. However, since paradoxical effects are often observed with supraoptimal doses of these compounds, the possibility of an immunodepression rather than of a stimulation could also be discussed. In order to obtain at least preliminary information on this problem the therapeutical activity of the *C. parvum*–Leva association was investigated in 2 murine experimental tumors.

The Lewis lung (3LL) carcinoma was maintained and transplanted in compatible C 57Bl/6 male mice; animals were sacrificed 25 days after the i.m. transplant of 2×10^5 line tumor cells and pulmonary metastases were evaluated by the technique of Wexler [5]. The P 815 mastocytoma was maintained in syngeneic DBA/2 mice and 10^6 cells transplanted s.c. in the footpad of compatible B6D2F1 male mice. Animals were obtained from Charles River Italy, Calco, Italy and weighed 20–22 g at the start of the tests.

The dose of *C. parvum* (Wellcome Res. Labs., Beckenham, England) and that of Leva (Janssen Pharma, Beerse, Belgium) employed were 0.7 mg/mouse i.v. and 3 mg/kg i.p., respectively. Both doses have repeatedly been shown to exert optimal antitumoral effects in a large number of mouse cancer systems [2, 6, 7]. Each experimental group consisted of at least 10 animals and the statistical analysis was performed by the Duncan's New Multiple Range Test and Mann-Whitney U. Test. As shown in Table 1 by one representative experiment of the 6 performed, a single injection of *C. parvum* on day 13, i.e. when the primary tumor weight was in the 1.8–2 g range, produced a significant decrease in both the number and weight of pulmonary metastases of 3LL-bearing mice. Conversely, single doses of Leva administered on day 7, 14 or 19 were not significantly effective in these conditions (results are reported only from animals treated with Leva on day 7). When Leva (day 7) preceded *C. parvum* injection on day 13, the activity obtained was equivalent to that seen with the latter agent alone, whereas a significantly greater antitumoral effect was observed when treatment with Leva followed *C. parvum* either by 1 or 6 days. Table 2 presents representative data obtained in the s.c. P 815 mastocytoma model; it may be observed that the treatments of Leva or *C. parvum* alone employed did not significantly prolong survival in these conditions, whereas the combination induced clear increases in lifespan over controls with both time intervals between *C. parvum* and Leva tested. However it is note-

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Table 1. *Effects of* C. parvum-*Leva combination on* 3*LL-bearing mice**

Exp. group	Day of treatment	Metast. weight (mg)	Metast. number
Controls	—	134.4±35.2	25.5±5.1
C. parvum	+13	66.1±17.8†	14.2±3.1†
Leva	+ 7	128.2±21.3	26.2±4.8
Leva	+ 7		
+		67.7±18.1†	13.1±2.8
C. parvum	+13		
C. parvum	+13		
+		12.5± 7.6‡	4.1±3.6‡
Leva	+14		
C. parvum	+13		
+		24.1± 5.8§	7.1±3.1§
Leva	+19		

*2×10^5 cells transplanted i.m. on day 0; *C. parvum* 0.7 mg/mouse i.v.; Leva 3 mg/kg i.p.
†$P < 0.05$ vs controls.
‡$P < 0.05$ vs *C. parvum*.
§$P \leqq 0.05$ vs. *C. parvum*.
Values represent the mean of 10 mice/group ±S.D. of the mean.

Table 2. *Effects of* C. parvum-*Leva combination on P*815 *mastocytoma bearing mice**

Exp. group	Day of treatment	MST	T/C
Controls	—	27.3±1.2	—
C. parvum	+ 2	31.6±1.9	115.8
Leva	+10	27.1±1.9	99.3
Leva	+20	28.0±2.1	102.6
C. parvum	+ 2		
+		36.3±1.3†	133.0
Leva	+10		
C. parvum	+ 2		
+		39.8±5.4†	145.8
Leva	+20		

*10^6P815 mastocytoma cells transplanted s.c. on day 0; *C. parvum* 0.7 mg/mouse i.v.; Leva 3 mg/kg i.p.
†$P < 0.05$ vs *C. parvum*.
Values represent the mean of 10 mice/group ±S.D. of the mean.

worthy that when 10^4–10^6 P815 mastocytoma cells were transplanted i.p. in the same strain of mice no increase in the survival time was determined by the administration of *C. parvum* and Leva alone or in combination using different schedules.

These data thus show that the combination of *C. parvum* and Leva, both known immunomodulators, can result in increased antitumoral and antimetastatic activity although the experimental conditions seem to play a critical role for the evaluation of these effects.

The mechanism(s) at the basis of this synergistic activity are still unknown. More specifically, it is still undetermined whether the better antitumoral effect seen in these conditions was merely the result of a higher level of immune stimulation than attainable by either drug alone although used in optimal doses as in these experiments, or was the consequence of the stimulation of at least partially independent immune effector mechanisms acting synergistically. It is suggestive in this regard that main target of Leva is believed to be the T lymphocyte [7, 8], a cell lineage whose function is generally depressed by *C. parvum* [9]; the former agent is also generally regarded as acting more as a restorer of a depressed immune capacity rather than as a true immunostimulant [10]. These data however are still too limited to allow conclusions on these points and also at the light of recent findings showing that transient agranu-

locytosis can be associated to Leva administration in man [11], a larger number of experimental studies should be performed before evaluating the therapeutic potential of this agent in combination with other immunostimulants or chemotherapy.

Finally it should be mentioned that this favorable combination could be also related to an interference of *C. parvum* on the metabolism and/or distribution of Leva since it is known that the former agent inhibits liver microsomal enzymes [12].

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NEWS FROM E.O.R.T.C.

The E.O.R.T.C. Data Center

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1. INTRODUCTION

IN RECENT years there has been an important increase in the number of clinical trials undertaken by the E.O.R.T.C.* clinical groups. The volume of data has become so large that it was felt necessary to have a central body in order to maintain a maximum amount of uniformity in design, follow-up, reporting and analyses. There are currently 23 groups actively engaged in clinical cooperative work. There are, as of 1 May, 1977, 64 clinical studies in progress under the supervision of the E.O.R.T.C. Council [1]. Most of these studies are monitored by the E.O.R.T.C. Data Center whose activities is the design and review of new clinical trials, the design of data forms, data collection, management and storage and final statistical analyses are described in this paper. About 1000 new cases are randomized every year in E.O.R.T.C. studies. A total of approximately 4200 patients from 130 different institutions in 13 countries have so far been entered into cooperative group studies handled by the Data Center.

2. STRUCTURE OF THE DATA CENTER

The E.O.R.T.C. Data Center is under the supervision of the E.O.R.T.C. Council to whom it reports its activities once a year. It is to-day composed of a Director,† 2 senior statisticians, ‡ 1 computer analyst,§ 1 programmer, || 4 data managers¶ and 2 medical secretaries.**

3. THE DESIGN OF A COOPERATIVE TRIAL

A clinical trial is to be considered as a human experimentation limited by ethical considerations. There must be experimental facts and logical arguments indicating that the new modality of treatment might be better than any known therapy for all patients included in the trial. When such a proposition can be made, it is imperative to design the study in such a way that generalization of the conclusions to other patients with the same disease will be possible.

The statistical staff of the E.O.R.T.C. Data Center work with the cooperative groups in the review of new and standard therapies, in selection of prognostic variables for stratification and covariate measurements and in the determination of sample sizes and the duration of the trial.

The design of a trial begins with a review of the principal endpoints and a listing of known prognostic variables for patient response. Depending on what is known about the statistical distribution of the endpoint responses and how they are related to the prognostic variables, one can determine which variables must be used for patient stratification, what will be the most efficient method known for analyzing the data, and either how sensitive a trial of fixed size will be to therapy differences of given orders of magnitude or, for specified minimal differences, how large a trial will have to be to achieve the sensitivity needed to detect these differences.

4. THE PROTOCOL

Once a protocol has been written by an E.O.R.T.C. group, it is submitted to the Data Center who, in conjunction with the Protocol Review Committee, checks it for accuracy, adds paragraphs concerning the registration and

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†Maurice J. Staquet, ‡Richard Sylvester, David Machin, §Martine van Glabbeke, ||Georgette De Grauwe, ¶Ann Wennerholm, Jane Tyrrell, Josette Renard, Marleen De Pauw, **Dominique Eeckhoudt and Julie Tyrrell.

randomization of patients, the number of patients required, the expected duration of the study and the schedule for submission of forms. The final protocol must contain the following items before distribution to the Protocol Review Committee for final approval:

1. Background and introduction.
2. Purpose of the study.
3. Selection of patients.
4. Design of the study, including a schema.
5. Pretreatment studies.
6. Therapeutic regimens.
7. Toxicity.
8. Follow-up and duration of the study.
9. Criteria of evaluation.
10. Registration and randomization of patients.
11. Submission of forms.
12. Statistical considerations.
13. Administrative responsibilities.
14. References.

Appendices: Performance status scales, TNM classifications.

5. DESIGN OF FORMS

Once the protocol has been finalized, appropriate forms for the study are designed by the data manager together with the study coordinator, statistician and computer analyst. In general, each group requires its own individual set of forms and procedures, but the data forms, the structure of the data files and routine procedures for handling the data for all groups, are standardized as much as possible.

With some modifications the following basic forms are used by all groups:

(i) The registration form (Form I)
(ii) The on-study form (Form II)
(iii) The flow sheet (Form VII)
(iv) The summary form (Form IX)
(v) The progress report form (Form III)

Some groups may require additional forms such as:

(i) Radiotherapy and/or chemotherapy form (Form IV)
(ii) Surgical report form (Form V)
(iii) Measurement form (Form VIII)

Once the forms for a study are designed and typed by the Data Center, they are put into use for a trial period of several months in order to remove any final ambiguities and deficiencies before the final printing.

6. RANDOMIZATION

The basic feature of a randomized clinical trial is the random assignment of patients to different treatment groups. One of the treatment groups is usually a control group. Controls may consist of patients receiving no treatment at all, a standard treatment, or patients receiving the same treatment but a different dose or according to a different schedule. Using a control group chosen by any method other than by randomization requires the assumption that either the control and treatment groups are identical with respect to all important variables except the treatment under study, or that one can correct for any such differences (this assumes that all factors affecting the prognosis are known). Because our knowledge of those factors which may affect response to treatment is always incomplete, randomization is used as a method of assigning treatments to patients which permits valid statistical inferences to be drawn without any special assumptions.

There are three major advantages to randomization:

1. Bias is eliminated from the assignment of treatments.
2. Randomization tends to balance treatment groups with respect to factors related to the patients' prognosis whether or not these factors are known. Different treatment groups must be as similar as possible with regards to all possible factors so that after treatment, if differences in survival or length of remission do exist, these differences can be attributed to the treatment itself.
3. Randomization guarantees the validity of the statistical tests of significance which are used to compare treatments.

For randomized trials a random log must be created before patients can be entered on-study. The log is usually designed in such a way that treatments are balanced between patients from a participating institution and also balanced among important prognostic variables or strata as specified in the protocol. Such a log is created by a standard computer program which allows for the particular features of an individual study.

Randomizations are usually made by institutions telephoning the data managers' office at the Data Center, but can also be made by telex, telegram or letter. The first step in registering a patient for a particular study is for the data manager to check with the caller that the patient satisfies the eligibility criteria specified in the protocol. Once this preliminary check is made, the name of the patient is entered on the next space in the random log under the appropriate institution and stratum. The treatment assigned is then read from the random log and given to the caller. At this stage a "registration form" is completed by the data manager.

The physician in charge of the patient then completes an "on-study form" which contains basic patient information such as previous history, description of the disease and the treatment group assigned by randomization, and sends it to the Data Center, normally within one week of registration. The on-study form serves as a second check as to whether the patient is eligible to enter the study and whether the correct treatment is being given.

7. DATA RECEIPT AND MANAGEMENT

Once a randomization has been completed, a patient file is created by the data manager, in which copies of all forms, correspondence and information concerning a particular patient is kept. Naturally all records are treated strictly confidential. First steps are also taken to create a computer file: each patient is identified by a 15-digit code assigned at the time of registration. This code identifies the particular study, the participating institution, the date of patient registration and the sequence number.

Once a registration has been completed and the patient is "on-study", information is collected periodically on treatment forms and/or flow sheets as long as the patient remains "on-study". These forms are returned to the Data Center at specified intervals and indicate if the treatment has been given in accordance with the protocol, if side effects have been encountered, if consequent dose modifications have taken place and if laboratory and physical examinations have been performed. When a patient goes "off-study", usually either because the treatment period as specified in the protocol has ended, progression, death, or loss to follow-up, a summary form is returned to the Data Center specifying the reasons.

The most important function of the data manager is to review all patient forms that are received for drug toxicity, ambiguities, completeness and protocol violations. In addition, she must also send requests to institutions for overdue forms. This work involves close contact with the participating physicians and detailed knowledge of the individual protocols.

After the completed forms have been received and checked by the data managers, the data are then sent to be punched on cards, either directly from the forms themselves or from coding sheets prepared by the data managers. The punched cards, each of which contains in its first 15 columns the patient code already referred to, are ready to be read by and stored on the computer for eventual recall and processing. Each time a new form is received, it is added to the computer file after verification by the data manager and is further verified for accuracy by the computer itself.

8. PREPARATION OF ADMINISTRATIVE REPORTS

Each E.O.R.T.C. cooperative group meets twice a year. At these meetings, the Data Center presents interim administrative reports and statistical analyses when appropriate. The administrative reports usually include the following information listed separately for each participating institution: total number of patients registered on a particular trial, the numbers in each of the treatment arms, summaries of any toxicities observed, reasons for patients going "off-study" and the number of "overdue" forms.

9. THE COMPUTER

The Data Center uses two large and powerful C.D.C. computers, located at the Université Libre de Bruxelles, for storing, verifying and processing the patient data received. The Data Center requires the use of such computers for two main reasons:

1. The large quantity of data that must be stored and processed.
2. The complexity of the statistical techniques required to analyze the data.

The computer also allows speedy access to the print-out of information for any individual patient, while the graph plotter can produce survival curves in a clear and readily reproducable format.

All patient information received by the Data Center is stored on computer files. For every E.O.R.T.C. study, there is a computer data file, stored on a magnetic disc. Magnetic tape copies of these discs are made as a precaution against loss of data caused by computer breakdowns . . . or human failures. The structure of the data files is completely and clearly described in a special library file called "variable description file".

10. VARIABLE DESCRIPTION FILE

Before the start of any study, the data managers write a "variable description file". This file is a detailed description of the exact piece of information that is to be found on each card of a patient's computer file; for example, the 17th column of every card gives the number of the data form. The variable description file works in the following way: all "on-study forms"

usually contain information on the weight of a patient. If the study involves only mature male patients, then the probable range of weights might be from 50 to 100 kg. This range of weights is included in the "variable description file". The weight of any new patient entered on study is checked against the above range and an error message is printed out by the computer if it is outside this range. The attention of the data manager is then drawn to this item of information and she may find on checking, either a transposition error from the form to the punched card or a physician error in recording the weight. It is only when the correct information is available that the patient record is accepted or put on the computer file. More importantly the "variable description file" is written to examine and verify the most important criteria for a patient's eligibility to enter a particular study.

11. ACCESS TO THE COMPUTER FILES

Access to the computer files can be made in several ways:

(i) By a punch card reader: the cards themselves contain the basic data and/or directions for the computer to execute a particular procedure. The computer "replies" by printing out on a fast printer, usually after some delay. A deck of cards usually contains:
   1. Instructions for the computer (for example: a request that will allow the introduction of some patient information into a computer file).
   2. Data cards (for example: the new information to be introduced).

(ii) By a screeen terminal: instructions or data can be typed on the screen and sent directly to the computer. The results appear either on the screen itself or are directed to a printer, or both. The screen allows one to work in "conversational mode" with the computer giving rapid replies to keyboard instructions. Such a mode allows immediate corrections and amendments to files, retrieval of data and results of statistical analyses (at the speed of 900 characters/sec).

(iii) By a portable teletype, linked to the computer by means of any telephone receiver. This works in the same way as the screen terminal except that results are printed on paper (at the speed of 30 characters/sec).

12. PROCESSING OF THE DATA

Once information has been stored on the computer, it is essential that the computer is able to sort, count, analyze and print summary tables of the data as rapidly as possible. The E.O.R.T.C. Data Center has a system of programs similar to those used in Buffalo, NY, but which have been adapted and extended to the E.O.R.T.C. data processing problems.
The main features of this system are:

1. The data management programs.
   These include:
   (i) The "RANDOM" program that organizes the printing of the random logs, and balances treatments within strata and within participating institutions.
   (ii) The "UPDATE SYSTEM", a system of programs that checks new data and rejects the wrong ones, updates the data files on discs and prints out information helping the data managers to control the status of their computer files. Most of the programs of the "UPDATE SYSTEM" can be used in conversational mode.
   (iii) The "VARIABLE DESCRIPTION FILE UPDATE SYSTEM"..
2. The administrative programs.
   These include:
   (i) The "ADMIN" programs accessible by conversational procedure allows the print out of selected tables, either by study or by institution. For example:
      (a) allocation of treatment by institution;
      (b) patients "on-study", "off-study";
      (c) request of missing forms;
      (d) response to the treatment;
      (e) toxicity by treatment;
      (f) stratification (type of surgery ...);
      (g) sex (response to treatment);
      (h) age (response to treatment);
      (i) type of surgery, etc. ...
      The "ADMIN" program also allows print out of the complete patient record (treatment, randomization date, stratification, etc. ...).
   (ii) The "QUEST" program that allows one to retrieve any set of data for all the patients of a study. This program is accessible by a conversational procedure.
   (iii) The "REQUEST" program that prints forms requested from the computer file.
   Other administrative programs are printing out tables and information specific to particular studies.

3. The statistical programs.
   These include programs to estimate and plot survival (and remission) curves for individual treatments and to test for differences in survival or free interval between different groups of patients.
   Regression programs allow one to assess the influence of prognostic variables by use of different statistical models.
4. The mathematics and numerical analysis programs.
5. The system management programs.
   These are helping to manage discs, tapes and program libraries.

13. THE STUDY COORDINATOR FILE

Copies of each patient's data forms are collected during a trial and placed in a separate file for the study coordinator. Each form in this file is screened by the study coordinator (sometimes a committee is formed for this purpose) and a final decision is made concerning whether a patient is eligible and can be included in the final analysis. Reasons for patient exclusion at this stage vary but might be for example, a protocol violation which is beyond the investigator's control. It is important to stress that this final screening of the data is in the hands of the clinicians directing the trial and is of paramount importance if the published results of the trial are to have validity. It should be noted that no such rigorous screening is performed before interim reports so that care has to be taken not to be unduly influenced by trends suggested by data not so verified.

14. THE FINAL STATISTICAL ANALYSIS

The final analysis contains a detailed review of all aspects of the trial including:

(i) admintrative problems in running the trial;
(ii) verification of initial prognostic variables and examination of potentially new prognostic variables;
(iii) analysis of the principal endpoints of the trial as set out in the protocol.

13. CONCLUSION

In the 3 years that the E.O.R.T.C. Data Center has been in operation, the groundwork has been laid for the provision by an organization devoted full time to E.O.R.T.C., of a wide range of statistical and data processing capabilities oriented towards the special problems arising from cooperative clinical trials.

Perhaps, the primary achievement of the Data Center has been the development of close working relationships with 23 cooperative groups. The total cost and effort involved in developing such capabilities for each group in 13 different countries (Table 1) would have far exceeded the actual cost through the Data Center.

Table 1.

Participating countries
1. Austria
2. Belgium
3. Canada
4. Czechoslovakia
5. Denmark
6. France
7. Germany
8. Italy
9. Kuwait
10. The Netherlands
11. Spain
12. Switzerland
13. United Kingdom

The goal of improving the quality of studies has been achieved (we believe) through the help provided in the planning of studies, the standardization of data handling procedures, the provision of efficient data processing techniques and sophisticated statistical analyses. Further improvements are anticipated through an increased awareness on the part of clinical investigators of the value of a reliable and experienced Data Center devoted to the special problems of clinical research.

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Announcements

EUROPEAN PHARMACOKINETICS

E.O.R.T.C. is planning a meeting on pharmacokinetics and metabolism of anticancer agents at the beginning of next year. Investigators interested in this problem are asked to get in touch with Dr. M. Staquet, E.O.R.T.C. Coordinator, Institut Jules Bordet, rue Héger-Bordet 1, 1000 Bruxelles, Belgium (phone: 538.57.90).

FUNDAMENTAL AND PRACTICAL ASPECTS OF THE APPLICATION OF FAST NEUTRONS IN CLINICAL RADIOTHERAPY

A 3rd International Meeting on: "Fundamental and practical aspects of the application of fast neutrons in clinical radiotherapy" will be held on 13–15 September, 1978 in The Hague, sponsored by the Organization for Health Research TNO, The Netherlands.

The purpose of this meeting is firstly to evaluate the clinical experience obtained in recent years about responses of tumours and normal tissues irradiated with high LET radiation, in comparison with responses to conventional types of radiation. Secondly a discussion will be held about the significance of various radiobiological and physical factors which influence the magnitude of possible advantages of high LET radiation. Finally a discussion of protocols for clinical trials is foreseen. This is of special importance because at present protocols of cooperative trials are developed, in the U.S.A. supported by the National Cancer Institute and in Europe by the Neutron Therapy Project Group of the European Organization on Research and Treatment of Cancer.

The meeting can be attended by all who are actively engaged in the clinical application on high LET radiation and in research connected with the subjects described. Presentation of papers will be by invitation only, except that a limited part of the time will be allotted to short communications or to poster sessions about new special developments and results. These communications may have to be limited by the organizing committee.

Registration forms and further information can be obtained from Miss M. C. von Stein, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk, The Netherlands.

G. W. Barendsen, J. J. Broerse and K. Breur
Co-organizers of the Meeting

Europ. J. Cancer Vol. 13, pp. 1462–1463. Pergamon Press 1977. Printed in Great Britain

Recent Journal Contents (1977)

International Journal of Cancer — *November, 1977*

Human Cancer

N. C. Nayak, A. Dhar, R. Sachdeva, A. Mittal, H. N. Seth, D. Sudarsanam, B. Reddy, U. L. Wagholikar and C. R. R. M. Reddy: Association of human hepatocellular carcinoma and cirrhosis with hepatitis B virus surface and core antigens in the liver.

P. A. Trumper, M. A. Epstein, B. C. Giovanella and S. Finerty: Isolation of infectious EB virus from the epithelial tumour cells of nasopharyngeal carcinoma.

W. Henle, J. H. C. Ho, G. Henle and H. C. Kwan: Nasopharyngeal carcinoma: significance of changes in Epstein–Barr virus-related antibody patterns following therapy.

R. S. Kerbel, H. F. Pross and A. Leibovitz: Analysis of established human carcinoma cell lines for lymphoreticular-associated membrane receptors.

N. Breslow, C. W. Chan, G. Dhom, R. A. B. Drury, L. M. Franks, B. Gellei, Y. S. Lee, S. Lundberg, B. Sparke, N. H. Sternby and H. Tulinius: Latent carcinoma of prostate at autopsy in seven areas.

K. Nishioka, M. N. Romsdahl, H. A. Fritsche, Jr. and M. J. McMurtrey: Tyrosine activity in the sera of patients with malignant melanoma: method and specificity.

B. Christenson: Complement-dependent cytotoxic antibodies in the course of cervical carcinoma.

L. C. Andersson, C. G. Gahmberg, K. Nilsson and H. Wigzell: Surface glycoprotein patterns of normal and malignant human lymphoid cells. I. T cells, T blasts and leukemic T cell lines.

K. Nilsson, L. C. Andersson, C. G. Gahmberg and H. Wigzell: Surface glycoprotein patterns of normal and malignant human lymphoid cells. II. B cells, B blasts and Epstein–Barr virus (EBV) positive and negative B lymphoid cell lines.

T. Ø. Landaas, T. Godal and T. B. Halvorsen: Characterization of immunoglobulins in Hodgkin cells.

J.-P. Lamelin, R. Ellouz, G. de-Thé and J. P. Revillard: Lymphocyte subpopulations and mitogenic responses in nasopharyngeal carcinoma, prior to and after radiotherapy.

Experimental Cancer

M. Hayami, J. Ignjatovic and H. Bauer: Avian retrovirus-induced surface antigens and their cross-reactivity with chemically transformed cells and primary embryonic cells of Japanese quails.

C. Pietropaolo, N. Yamaguchi, I. B. Weinstein and M. C. Glick: Glycopeptides from epithelial cell mutants temperature sensitive for the transformed phenotype.

R. C. Harmon, E. A. Clark, A. L. Reddy, W. H. Hildemann and Y. Mullen: Immunity to MCA-induced rat sarcomas: analysis of *in vivo* and *in vitro* results.

J. Calafat, K. Weijer and H. Daams: Feline malignant mammary tumors. III. Presence of C-particles and intracisternal A-particles and their relationship with feline leukemia virus antigens and RD-114 virus antigens.

J. R. Connell and C. H. Ockey: Analysis of karyotype variation following carcinogen treatment of Chinese hamster primary cell lines.

H. F. Stich, A. B. Acton, B. P. Dunn, K. Oishi, F. Yamazaki, T. Harada, G. Peters and N. Peters: Geographic variations in tumor prevalence among marine fish populations.

J. K. Youn, M. Santillana, G. Hue and G. Barski: Virus expression in different tissues of normal and tumor-bearing mice inoculated with a murine leukemia virus.

E. R. Phillips and J. F. Perdue: Immunologic identification of fetal calf serum-derived proteins on the surfaces of cultured transformed and untransformed rat cells.

E. D. Crum and D. D. McGregor: Induction of tumor resistance with BCG-associated tumor antigen.

International Journal of Cancer *December, 1977*

Human Cancer

R. J. Jamasbi and P. Nettesheim: Increase in immunogenicity of a pulmonary squamous cell carcinoma, propagated *in vitro*.

R. Fäldt and J. Ankerst: Tumor associated humoral cytotoxicity in patients with acute myelogenous leukemia before and after chemotherapy.

M. J. Lopez and D. M. P. Thomson: Isolation of breast cancer tumour antigen from serum and urine.

J. Spira, S. Povey, F. Wiener, G. Klein and M. Andersson-Anvret: Chromosome banding, isoenzyme studies and determination of Epstein–Barr virus DNA content on human Burkitt lymphoma/mouse hybrids.

R. MacLennan, J. Da Costa, N. E. Day, C. H. Law, Y. K. Ng and K. Shanmugaratnam: Risk factors for lung cancer in Singapore Chinese, a population with high female incidence rates.

A. B. Rickinson, S. Finerty and M. A. Epstein: Mechanism of the establishment of Epstein–Barr virus genome-containing lymphoid cell lines from infectious mononucleosis patients: studies with phosphonoacetate.

J. D. Rowley, H. M. Golomb, J. Vardiman, S. Fukuhara, C. Doughterty and D. Potter: Further evidence for a non-random chromosomal abnormality in acute promyelocytic leukemia.

S. H. Golub, D. M. Rangel and D. L. Morton: *In vitro* assessment of immunocompetence in patients with malignant melanoma.

C. Desgranges, J. Y. Li and G. de Thé: Secretory nature and tumorous origin of IgA antibodies to Epstein–Barr virus in saliva of nasopharyngeal carcinoma (NPC) patients.

H. E. Heier, N. Carpentier, G. Lange, P. H. Lambert and T. Godal: Circulating immune complexes in patients with malignant lymphomas and solid tumors.

B. M. Vose, F. Vánky and E. Klein: Human tumour-lymphocyte interaction *in vitro*. V. Comparison of the reactivity of tumour infiltrating, blood and lymph node lymphocytes with autologous tumour cells.

J. Taylor-Papadimitriou, M. Shearer and M. G. P. Stoker: Growth requirements of human mammary epithelial cells in culture.

E. H. Larner and C. L. Rutherford: A microchemical analysis of alkaline phosphatase in human malignant and benign breast tumors.

Experimental Cancer

R. T. Prehn: Immuno-stimulation of chemical oncogenesis in the mouse.

M. V. Pimm and R. W. Baldwin: *C. parvum* immunotherapy of transplanted rat tumours.

J. Okabe, Y. Honma and M. Hozumi: Inhibition of RNA acid and protein syntheses makes non-differentiating mouse myeloid leukemia cells sensitive to a factor(s) stimulating differentiation.

S. Ohno, J. Luka, L. Falk and G. Klein: Detection of a nuclear, EBNA-type antigen in apparently EBNA-negative, Herpesvirus papio (HVP)-transformed lymphoid lines by the acid-fixed nuclear binding technique.

G. G. Hatch, P. J. Balwierz, B. C. Casto and J. A. DiPaolo: Characteristics of hamster cells transformed by the combined action of chemical and virus.

G. Hugoson, B. Lagerlöf and B. Thorell: The effect of chronic protozoan infection by Babesia rodhaini on leukemogenesis in mice.

J. H. Dean, D. D. Lewis, M. L. Padarathsingh, J. L. McCoy, J. W. Northing, T. Natori and L. W. Law: Cellular immunity to SV40-induced tumor cells and solubilized tumor-associated antigens in immune mice using an isotopic footpad assay.

Europ. J. Cancer Vol. 13, p. 1464. Pergamon Press 1977. Printed in Great Britain

Papers to be Published

A. TROUET

Increased selectivity of drugs by linking to carriers.

M. CIKES

Expression of hormone receptors in cancer cells: a hypothesis.

Y. M. RUSTUM and D. J. HIGBY

Biochemical and clinical studies of chronic lymphocytic leukemia.

F. STENBÄCK and J. ROWLAND

Role of particle size in the formation of respiratory tract tumors induced by benzo(a)pyrene.

A. H. YRA and J. J. FENNELLY

Neuraminidase-like effect of vitamin A on cell surface.

I. GRESSER, C. MAURY and M. TOVEY

Efficacy of combined interferon-cyclophosphamide therapy after diagnosis of lymphoma in AKR mice.

M. FIORENTINO, G. PALU, P. SPERANDIO. O. VINANTE, P. DE BESI, G. REALDI and N. PENNELLI

Non-African sporadic Burkitt's lymphoma in Italian patients.

A. ČIHÁK

Transformation of 5-aza-2′-deoxycytidine-^{3}H and its incorporation in different systems of rapidly proliferating cells.

R. VERLOES, G. ATASSI, P. DUMONT and L. KANAREK

Tumor growth inhibition mediated by trypsin inhibitor or urokinase inhibitors.

C. DAVE, M. A. PAUL and Y. M. RUSTUM

Studies on the selective toxicity of guanazole in mice.

D. R. BUDMAN, E. CAMANCHO and R. E. WITTES

The current causes of death in patients with malignant melanoma.

J. L. BIEDLER, A. M. ALBRECHT and B. A. SPENGLER

Biochemical and karyological properties of cells resistant to the quinazoline antifolate, methasquin.

J. J. ROUSSEAU, G. FRANCK, T. GRISAR, M. RESNIK, G. HEYNEN and J. SALMON

Osteosclerotic myeloma with polyneuropathy and ectopic secretion of calcitonin.

A. S. MORRISON

Geographic and time trends of coffee imports and bladder cancer.