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# Heterokaryon Formation Between Alkaline Phosphatase Variants in a Human Heteroploid Cell Line*

G. SIMONI and L. DE CARLI
*International Laboratory of Genetics and Biophysics, Pavia Section, 27100 Pavia, Italy*

## INTRODUCTION

THE FUNCTION of different genomes in a common cytoplasm can be conveniently studied by the method of cell fusion *in vitro*. Suitable agents of such a fusion are viruses known for their capacities of inducing polykaryocytosis [1]. A considerable amount of work has already been done with artificial hybrids induced by viruses [2–5]. The experiments reported here are part of a more general program whose final purpose is to study the regulatory characteristics of the mammalian cells. The first step of such an investigation is the detection of heterokaryons between cell variants in a specific character susceptible to control. The character used in the present experiments is the enzyme alkaline phosphatase (AP). The changes in the activity of this enzyme have been thoroughly studied in mammalian cultured cells [6, 7]. A variation in the AP levels occurs also within a cell line among clonal derivatives. Induction and repression phenomena have also been described for the same enzyme in cultured cells [8]. A typical example is given by the EUE, a human heteroploid line, which shows high levels of AP and its subline E6D with enzymic levels at the limit of sensitivity of the determination methods. The histochemical staining allows an easy recognition of $AP^+$ and $AP^-$ cells (Figs. 1a and 1b). In the present experiments the above cell variants have been used with the purpose of revealing the enzymic pattern of the immediate products of fusion.

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## MATERIALS AND METHODS

*Cell cultures.* The EUE cell line with high levels of AP (specific activity*: 60–90) and the E6D subline with very low enzymic activity (specific activity: 0·1–0·2) have been maintained in our laboratory according to the methods already described [9]. The E6D subline kept in continuous culture for 3 years after the last reisolation did not show any reversion to the AP positive parental condition during this period. The Eagle's medium supplemented with calf serum 10% has been used in all cases.

*Virus characteristics.* The virus used in the present experiments is a strain of Sendai, kindly supplied by Dr. Koprowski of the Wistar Institute, Philadelphia. The virus has been propagated in our laboratory according to the method described by Harris and Watkins [2]. The titration of the viral suspension has been performed directly on the EUE cells. Serial two-fold dilutions of the infected allantoid fluid have been performed in drops of 0·03 ml, to which an equal volume of cell suspension has been added. The cell inoculum per drop was $10^4$. The agglutination unit has been defined as the smallest amount of virus particles still able to produce microscopically

*μmoles of *p*-nitrophenylphosphate hydrolyzed per min per mg of protein.

visible agglutination after 2 hr of incubation at room temperature (CAU).

The virus has been inactivated by incubating the undiluted virus suspension in the presence of β-propiolactone at a final concentration of 0·1% for 2 hr at 37° and thereafter by maintaining it for at least 18 hr at 4°.

*Histochemical staining and cytological preparations.* The histochemical procedure for revealing AP has been modified in order to allow a subsequent cytological staining, which shows the cell structures. To this purpose a modified Gomori's reaction has been coupled with May–Grümwald stain. The modification of the Gomori's method consisted in dissolving all the constituents of the reaction mixture (see the standard method [10]) in Hanks solution and treating the cells grown on coverslips with the combined solutions prior to fixation. After 10 min of incubation at room temperature the diazonium salt precipitates are visible inside the $AP^+$ cells. The coverslips are then washed three times in Hanks solution, fixed with methyl-alcohol for 5 min and stained with May–Grümwald solution for 5 min. The coverslips are washed again with water, air dried and mounted in Euparal. The counts of the nuclei of both $AP^+$ and $AP^-$ types have been performed directly under microscope at 400 magnifications. Multinucleated cells with no clear edges have been excluded from the scoring.

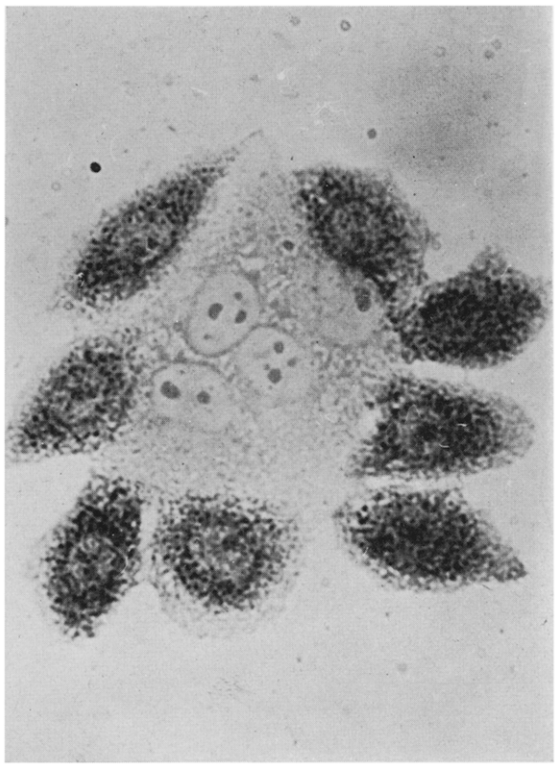
*Technique of fusion.* Cells from the two parental lines grown in monolayer cultures have been trypsinized with Difco trypsin 1:250, 0·25% in Hanks solution, dispersed in Eagle's medium for suspension cultures and incubated in hydrophobic plastic Petri dishes for overnight. The cells can be harvested from the plastic dishes by simply blowing the medium over with a pipette. The cells are then dispersed by pipetting them vigorously and resuspended in Hanks solution in an amount sufficient to bring the inoculum to $10^7$ cells/ml. Suspensions of the two types of cells have been obtained in this way. The cell mixture was made as follow: 0·5 ml EUE suspension + 0·5 ml E6D suspension + 1 ml virus suspension at titers ranging from 500 to 2000 CAU according to the various experiments. The cells treated with virus were then kept at 4° for 10 min and subsequently at 37° in horizontal agitation (100 excursions per min) for 30 min. At the end of incubation the suspension was diluted with growth medium and spun down at 800 rev/min for 5 min. The sediment was resuspended in fresh medium and the cells distributed into 60 mm Petri plates containing a coverslip. The inoculum was $3 \times 10^5$ cells per plate containing 5 ml medium. The cultures so made were kept in a $CO_2$ incubator for the periods required by the various experiments.

## RESULTS

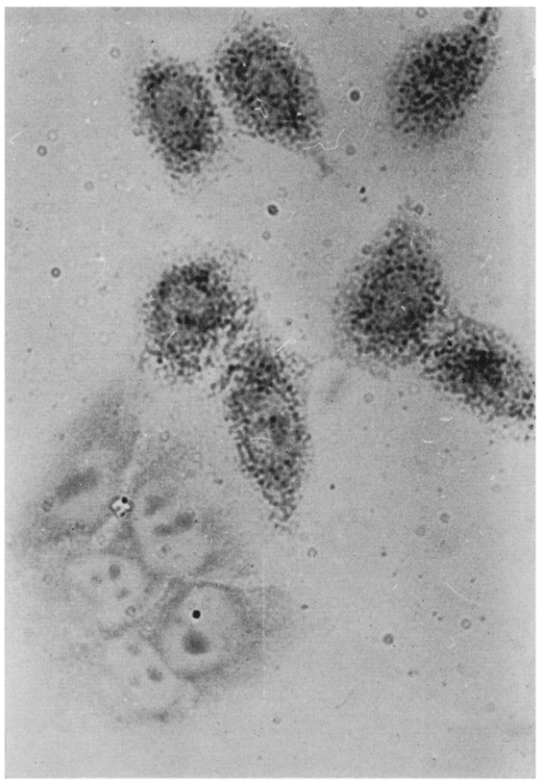
In preliminary experiments coverslips with adhering cells were withdrawn at 6, 12, 24, 48, 60 and 72 hr and treated with the histochemical staining followed by May–Grümwald stain. After 6 hr of incubation the cells were not flattened out enough as to allow an easy recognition of the nuclei. At 12 hr of incubation multinucleated cells were seen clearly. Between 12 and 24 hr most of the giant cells were showing two or more nuclei. In the subsequent times the proportion of polykaryocites decreases because they either die or transform into true hybrids (or 'synkaryons'). Therefore the 24 hr period has been taken as the most appropriate for determining the proportions of the two types of nuclei in the products of fusion. The slides have been scored for the proportions of $AP^+$ and $AP^-$ nuclei in mononucleated cells and in cells with two or more nuclei. Four classes of cells have been distinguished with 1, 2, 3 and more than 3 nuclei.

Within each class the $AP^+$ and $AP^-$ nuclei have been counted. The positive nuclei are easily recognizable as they are surrounded by a zone of cytoplasm showing a positive reaction for alkaline phosphatase (Figs. 1c, 1d and 2).

The fusion efficiency (FE) has been calculated from the ratio between the number of nuclei present in multinucleated cells and the total number of nuclei. In Table 1 are reported the nuclear counts made in one experiment with a high efficiency of fusion. The expected frequencies of cells with different number of nuclei have been calculated on the basis of the hypothesis of a random association of nuclei according to a Poisson distribution. The observed proportions do not fit with such a hypothesis, as indicated by the $\chi^2$ value. This is largely due to an excess of cells with a number of nuclei larger than 3. Furthermore the proportion of homokaryons versus heterokaryons appears to be larger than expected on the basis of a binomial distribution of the two types of nuclei within each class of cells. On the other hand the $AP^+$ and $AP^-$ nuclei distribute homogenously between mononucleated and multinucleated cells. Differences can be observed for specific combinations of nuclei but these are likely to be due to sampling errors. An heterogeneity test in a $2 \times n$ table has been performed by combining all the counts of $AP^+$ nuclei and those of $AP^-$ nuclei and classifying them as

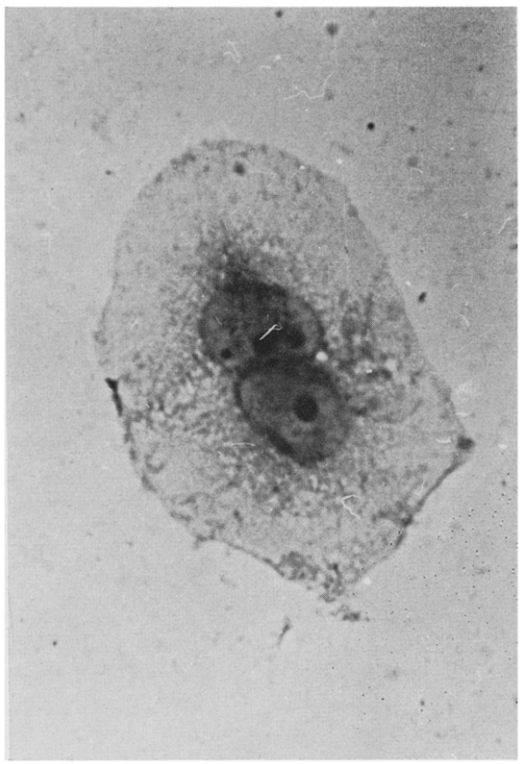


(*a*)



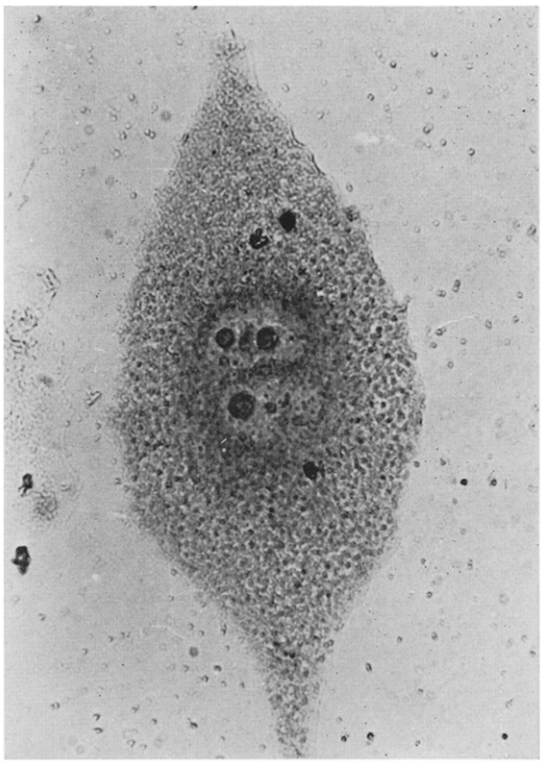
(*b*)

*Figs.* 1*a and b. Artificial mixture of EUE and E6D untreated cells. The EUE cells are darkly stained as a result of a positive reaction for alkaline phosphatase. The faint staining of E6D cells is due to May–Grümwald stain* (128 ×).



(*c*)

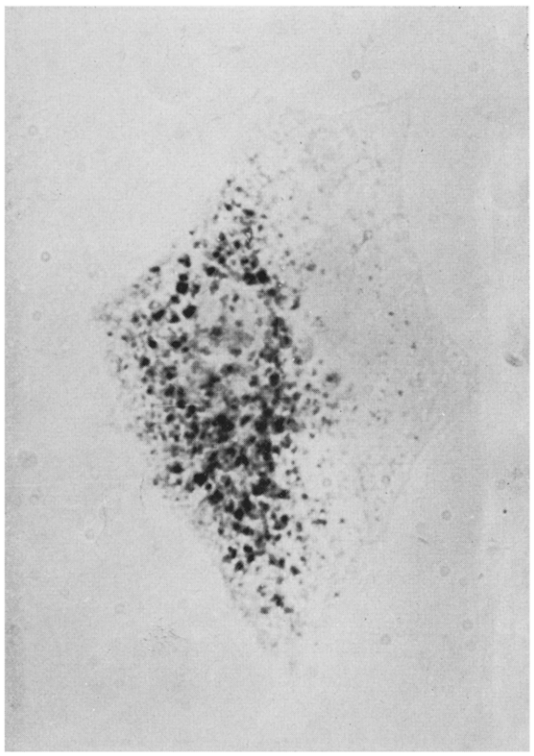
*Fig.* 1*c. Homokaryon of E6D cells, with two nuclei. Histochemical reaction negative + May–Grümwald stain* (128 ×).



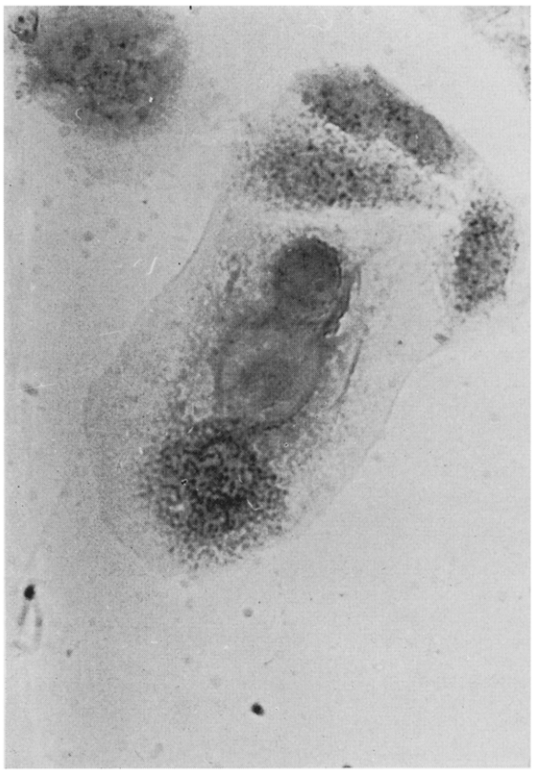
(*d*)

*Fig.* 1*d. Homokaryon of EUE cells, with two nuclei. Histochemical reaction positive + May–Grümwald stain* (128 ×).

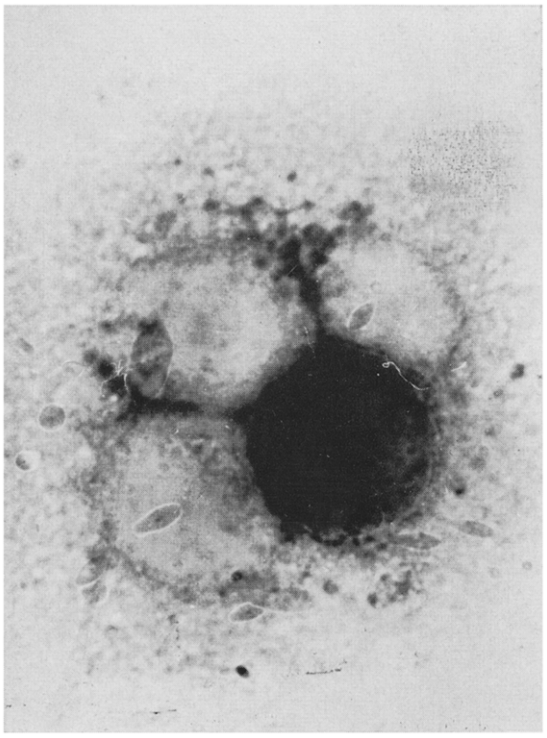
(to face p. 2)



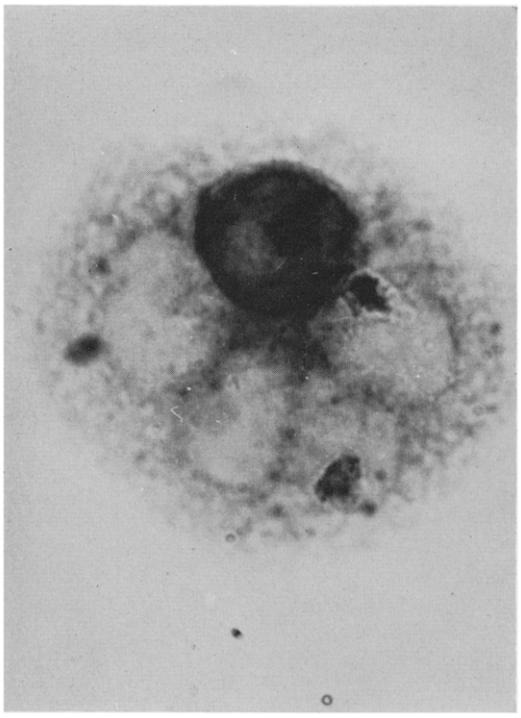
(*a*), 1 $AP^+$/1 $AP^-$;



(*b*), 1 $AP^+$/2 $AP^-$;

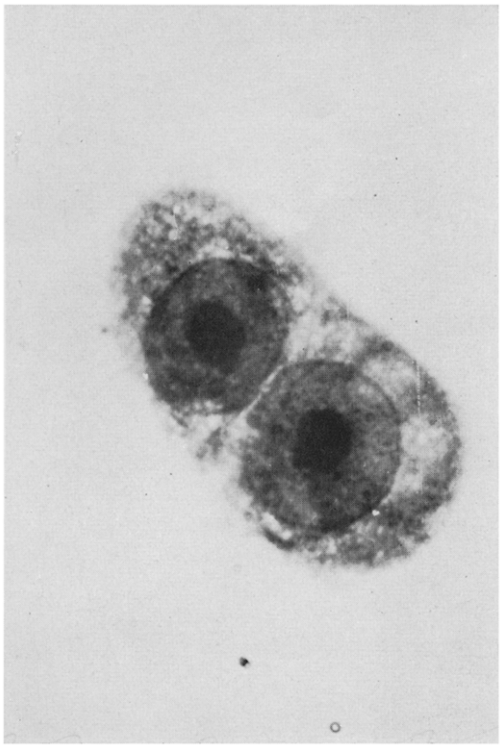


(*c*), 1 $AP^+$/3 $AP^-$;

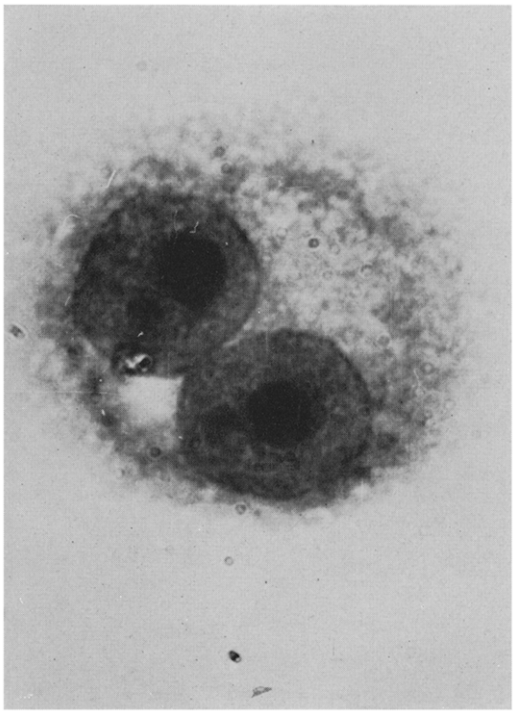


(*d*), 1 $AP^+$/4 $AP^-$.

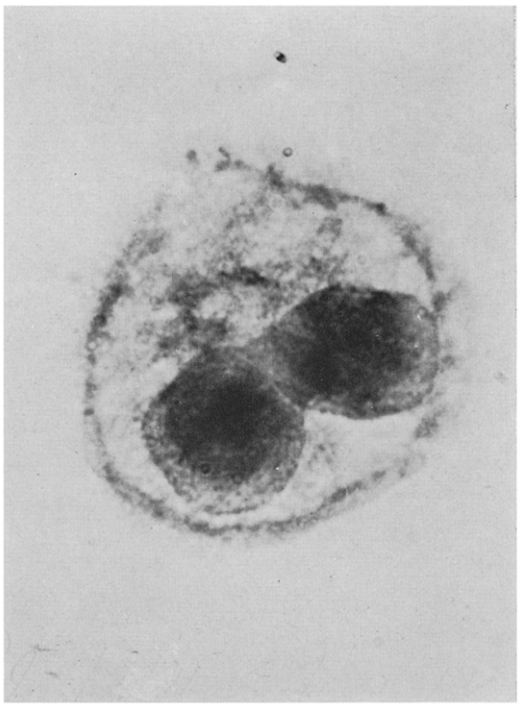
*Fig.* 2. *Heterokaryons with different number of* $AP^-$ *nuclei and* 1 $AP^+$ *nucleus. Histochemical reaction coupled with May–Grümwald stain in* (*a*) (320×) *and* (*b*) (128×); *histochemical staining coupled with acridine orange and preceded by fixation with* 95° *ethyl alcohol in* (*c*) *and* (*d*) (320×).



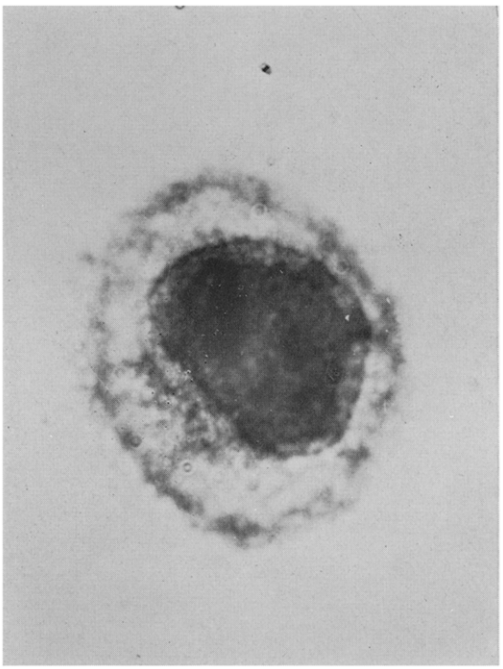
(*a*), *two separated nuclei in a binucleated cell;*



(*b*), *nuclei are connected through a small bridge of nuclear material;*



(*c*), *an advanced stage of nuclear fusion;*



(*d*), *the nuclei completely fused.*

*Fig.* 3. *Reconstruction of successive stages in the process of nuclear fusion. These images have been observed in a fusion experiment on preparations made* 48 *hr after the treatment with Sendai virus. Giemsas–May–Grümwald stain* (320×).

*Table* 1. *Distribution of AP+ and AP− nuclei in mononucleated and multinucleated cells in an experiment of cell fusion induced by Sendai virus*

| Total | No. of nuclei AP+ | AP | Observed | No. of cells Subtotal | Expected |
|---|---|---|---|---|---|
| 1 | 1 | 0 | 830 | | |
| | 0 | 1 | 807 | 1637 | 1506 |
| 2 | 2 | 0 | 160 | | |
| | 0 | 2 | 174 | | |
| | 1 | 1 | 74 | 408 | 580·7 |
| 3 | 3 | 0 | 33 | | |
| | 0 | 3 | 28 | | |
| | 1 | 2 | 26 | | |
| | 2 | 1 | 21 | 108 | 111·8 |
| >3 | >3 | 0 | 15 | | |
| | 0 | >3 | 18 | | |
| | 1 | 3 | 10 | | |
| | 2 | 3 | 3 | | |
| | 3 | 3 | 0 | | |
| | >3 | 3 | 0 | | |
| | 1 | >3 | 1 | | |
| | 2 | >3 | 1 | | |
| | 3 | >3 | 0 | | |
| | 3 | 1 | 5 | | |
| | 3 | 2 | 1 | | |
| | >3 | 1 | 4 | | |
| | >3 | 2 | 1 | | |
| | 2 | 2 | 6 | 65 | 19·5 |
| Total No. of cells | | | | 2218 | 2218 |

Parental lines: EUE unselected cell line with high AP levels
E6D clone with very low levels of AP
FE (fusion efficiency): 51·14
$\chi^2 = 169{\cdot}1$

belonging to mononucleated or multinucleated cells. The $\chi^2$ value points to an independency of the two criteria.

| | No. of nuclei | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | >3 | Total |
| AP+ | 830 | 394 | 167 | 138 | 1529 |
| AP− | 807 | 422 | 157 | 158 | 1544 |
| Total | 1637 | 816 | 324 | 296 | 3073 |

$\chi^2[3] = 2{\cdot}816$

## CONCLUSIONS

The observed deviations from a random association of nuclei in multinucleated cells can partly be attributed to technical factors and partly to the characteristics of the dynamics of the process of cell fusion. Among the technical factors it is worth considering the spontaneous cell aggregation favoured by the high inoculum densities. Such an aggregation might take place in the cell suspensions from the two parental cell lines before mixing. In such a case the excess of homokaryons over heterokaryons would be accounted for. On the other hand if the spontaneous aggregation occurs after cell mixing and before the agglutination induced by the virus, an excess of cells with larger number of nuclei would be expected. However, the transformation of the spontaneous cell aggregates in true polykaryocites does not seem too easy in view of the relative protection of cell clumps against the action of the virus.

As far as the factors intrinsic to the fusion process are concerned the following considerations can be made:

(a) as assumed by Harris and Watkins [2] the multinucleated cells are more resistant than the mononucleated ones to the cytotoxic action of the virus: such a selective advantage might exist also for varying degrees of multinuclearity. This could explain the excess of cells with more than three nuclei.

(b) A fraction of multinucleated cells with the same kind of nuclei could originate from the division or fragmentation of the nucleus

without cell division. Such a process, presumably also induced by the cytotoxic action of the virus, would bring about an excess of homokaryons.

(c) Cells with increasing number of nuclei might result from successive or serial fusions. If such a process takes place we would expect the number of nuclei per cell to increase with the time of the exposure to the virus as well as with the titer of the virus suspension.

At the present stage of the investigation it is not possible to establish which one(s) of the above factors are responsible for the observed deviations from a random association of nuclei.

The maintenance of the original proportions of $AP^+$ and $AP^-$ nuclei in multinucleated cells suggests that at least within the first 24 hr after fusion no apparent interaction exists between the two types of nuclei in their combined cytoplasms. The presence of a repressor-like substance or of an inhibitor of the enzyme activity in the $AP^-$ parental cells which underwent fusion, would result in an increase of the relative frequency of $AP^-$ multinucleated cells. Conversely the passive transport of the enzyme or the presence of an inducer or an enzyme activator in the $AP^+$ parental cells would bring about a relative excess of $AP^+$ multinucleated cells. The histochemical pattern of the heterokaryons at 24 hr after fusion indicates that the enzyme persists and therefore there is no inhibitor of its activity rapidly diffusible into the common cytoplasm, but the enzyme is confined to a region near to the $AP^+$ nucleus. Such a characteristic points to the high degree of compartmentation of the cells involved and to the tight binding of the enzyme to the cell structures. This latter property has been noticed in a study of some physico-chemical properties of AP in the same lines used in the present experiments of hybridization [11]. The intermingling of the $AP^+$ and $AP^-$ cytoplasms appears surprisingly slow as they maintain their individuality for a period at least corresponding to one doubling time of the parental cells under normal conditions. More relevant to the study of the control of the enzymatic synthesis are the successive stages in the development of the heterokaryons ending to the formation of a true hybrid. The possible occurrence of nuclear fusions in the present mating system is demonstrated by the sequence shown in Fig. 3. However an analysis of the enzymatic patterns of the hybrids requires the establishment of cell lines developed from the heterokaryons. Experiments in this direction are in progress.

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## SUMMARY

*The fusion between cells from a human line with high alkaline phosphatase levels ($AP^+$) and from a clonal subline deficient in the same enzyme has been induced with Sendai virus. The proportion of cells with various numbers of nuclei has been determined and the polynucleated cells have been scored for numbers of $AP^+$ and $AP^-$ nuclei with the aid of the histochemical staining. The heterokaryons are readily recognizable as they show the enzyme in a region of their cytoplasm near to $AP^+$ nuclei. A higher proportion of homokaryons over heterokaryons within each class of multinucleated cells have been noticed. However the overall distribution of $AP^+$ and $AP^-$ nuclei in the multinucleated cells reflects the original proportion of the two types of mononucleated cells indicating the absence of any interaction between the two genomes in the immediate product of fusion.*

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# Control Mechanisms in Cancer*

M. ABERCROMBIE

*Department of Zoology, University College London, Great Britain*

THE TERM 'control mechanism' is an extremely vague phrase unless it is carefully delimited. Any factor that under any circumstance may regulate the rate or the direction of some cell activity can find itself called a control mechanism, though some sort of feed-back in the control system is often thought of as an additional qualification. I shall restrict myself to interactions between non-malignant cells that in tissue cultures seem to play a part in regulating cell locomotion and the replication cycle, and that seem to be deficient in cultures of malignant cells. These interactions have in them an element of feed-back, and so I think for everyone would qualify as control mechanisms. A striking feature of them is that they seem to require contact between the interacting cells, that is to say an apposition close enough to involve some degree of physical adhesion. It is of course an attractive idea that the cells of animals, which *in vivo* and *in vitro* are so extensively linked up by mutual contacts, control each other through such a communication network; but, though it is not a new line of thought, it is now a fashionable one, and needs therefore to be looked at somewhat critically. In particular, since these are tissue culture mechanisms, their application to the whole organism has to be a qualified one.

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## CELL LOCOMOTION

In normal adult tissues the main problem in the control of locomotion that is relevant to malignancy is that the great majority of cells are usually not exercising their powers of locomotion; but that in certain conditions, notably in wound-healing, they do move, though temporarily and to a limited extent.

Tissue culture has some suggestions to make about this problem. Indeed it is possible to construct *in vitro* a close analogue to the stationary *in vivo* population that is set into limited motion by wounding. The cells of a monolayer of fibroblasts soon after it has formed are not translocating, though they are usually oscillating to some extent round their mean positions. If a cell-free pathway is scratched in such a monolayer, the cells at either side of the empty space start to move; they move in an oriented way into the space; and when they have occupied the space they come to rest again. Such an experiment was first done many years ago by Albert Fischer [1]. It has recently become popular in the study of the control of mitosis, since mitosis is also stimulated around the wound, and so its results have been amply confirmed [2, 3].

The control of locomotory behaviour exemplified by this experiment is readily explicable by contact inhibition of movement [4], a form of behaviour in which, when one cell collides with another, its locomotion in the direction that produced the collision is stopped, though locomotion in another, uninhibited, direction

can then be started. A cell in a continuous monolayer, surrounded by neighbours, is liable to be prevented by contact inhibition from moving appreciably in any direction. A wound makes it possible to move into the bare space, but only in that direction; and when the space is filled and the monolayer restored, the cells are automatically brought to rest again (which is the element of feed-back in the process). A good deal of experimental work has gone into this analysis, and I shall not attempt to cover this old ground. I would like to discuss merely one aspect, the possible nature of such contact inhibition.

A fibroblast moving on a plane surface is lead by a flattened expanse of cytoplasm, which may be called the leading lamella. This usually bears irregular ruffles at or near its front edge, and hence is often called the ruffled membrane. It may be regarded as the locomotory organ of the cell. Some minutes after the leading lamella of a fibroblast has collided with some part of another fibroblast, it changes its activity [5]. It ceases to move forward, it loses its ruffles and it contracts. This contraction affects a variable extent of the leading lamella. It may drag it back, sometimes snapping it entirely free (contact retraction [6]), sometimes drawing out strands of cytoplasm which remain still attached where the original contact had been made; in either case it is evident that the original contact had been a rather firm adhesion. Sometimes when the adhesion is strong the contraction does the reverse of parting the cells; it draws the two cells together a little [7] without breaking the adhesion. It seems likely that the tension present in a confluent monolayer of fibroblasts, recently measured [8], is the outcome of these contractions at points of contact; and so probably are the tensions found in tissues *in vivo* which are a somewhat neglected mechanism of form change in embryos and in wound-healing [9]. At present I think the simplest hypothesis is to regard the phenomena of contact inhibition that I have mentioned as all essentially *due* to this spasm of contraction, which paralyses the mechanism of movement and obliterates the process of ruffling. This may be the primary response of the cell to contact with another. A similar contractile response has been described in epithelial cells [10] which also show all the other phenomena of contact inhibition [11, 12]. Dunn (unpublished) has observed the contractile response in moving nerve fibre tips when they collide, and it seems likely therefore to be a widespread mode of behaviour.

The nature of the message received by one cell from another that induces the contraction and other responses is quite unknown. Visible contact is a requirement. No change in colliding fibroblasts has been observed until after visible contact has been made. The apposition is evidently close enough to be an adhesion. Tight junctions are present in cultured fibroblasts [13], but whether they develop quickly enough to be involved in contact inhibition is not known. Any theory involving exchange of substances must meet the point that corresponding parts of similar cells in the same state of movement inhibit each other.

Contact inhibition does not always occur when two fibroblasts collide. I have not seen a clear case of failure when the two leading lamellae collide; but when the front of one cell collides with the side of another movement may continue so that the cells cross over each other. This occurs particularly commonly when the substratum to which the cells are attached is one to which they adhere rather poorly, such as agar. In all such collisions that I have watched with maximum resolving power, it can be seen that the cell whose leading edge makes the collision passes under the 'ventral' surface, i.e. between cell and substratum, of the cell whose side is collided with. As the leading edge passes through this narrow space it may go into the contraction of contact inhibition and withdraw. The leading edge may even emerge at the other side of the overlying cell, and only then go into contractile spasm. The relative positions of the two cells involved in such an asymmetrical collision has been clearly shown [14] by using a Stereoscan electron microscope on cells whose behaviour was observed immediately before fixation. The leading lamella of a cell, the presumed locomotory organ, which is closely attached to the substratum, does not therefore apparently change its substratum when cells cross over each other as a result of a failure of contact inhibition. In what way the contact has been defective or the response has failed in these cases is not yet known, but since they are commoner when the cell adheres poorly to the substratum, the initial presumption is that the size of the gap between cell and substrate behind the leading lamella is the relevant point.

Some time ago [4], it was suggested that one of the possible explanations of contact inhibition between fibroblasts might be that the dorsal surface of a fibroblast is not sufficiently

adhesive to allow another fibroblast to move over it. This explanation has been adopted by Carter [15] and Rubin [16]. It is certainly true that when fibroblasts run into a border of an artificial substratum to which they cannot adhere, they are stopped, as Carter [15] found using methyl cellulose, and we have found using agar. In our experience however the contraction and cessation of ruffling does not occur when cells are stopped in this way, and the phenomenon therefore bears little resemblance to contact inhibition. Furthermore, the hypothesis is not adequate for those instances when typical contact inhibition happens to a cell as it is passing beneath another, and therefore is not attempting to change substrata. However, at least with certain kinds of cells, it seems possible that this hypothesis has still to be reckoned with. Barski and Belehradek [17] found that malignant cells are conspicuously held up in their migration by cells which they considered were endothelial. We have found the same; both macrophages and mouse sarcoma S180, which are not inhibited in the ordinary way by collision with fibroblasts, are totally unable to move across cells originating from chick heart explants which we believe to be endothelial. Since endothelial cells in the organism, when not taking part in an inflammatory reaction, appear to be very non-adhesive, it seems possible that we may here be dealing with a situation analogous to the methyl cellulose or agar border already described. One wonders whether keratinised epithelium may not inhibit normal epithelium in this way [11]. The mechanism may indeed be potentially present, though masked by the contractile response, in fibroblasts and epithelial cells. At the least it is evident that this possibility has to be kept in mind. Contact inhibition, defined as the cessation of locomotion on contact, in the direction that has led to the contact, may therefore be of two sorts: that due to contact paralysis [18], and that due to slipperiness of cell surfaces. There may indeed be other mechanisms.

The theory of contact inhibition can, as we have seen, account for several aspects of the control of cell movement in culture. One would like to extrapolate the theory to the control of cell movements *in vivo*. There are good grounds for doing so in the case of epithelia, which can be directly observed in the organism. The idea that epithelia, moving during wound healing, stop moving when they collide with an opposing epithelial sheet, long antedates the term contact inhibition [19]. For other kinds of cells direct evidence is not really available. It is however a plausible suggestion that the appropriate orientation and stopping of other moving cells during wound healing in the whole organism is to be ascribed at any rate in large part to contact inhibition. But in the case of the tissue culture wound I also ascribed the *starting* of cell movement to the *lifting* of contact inhibition. It is necessary to mention at this point that something more is involved in the starting of cell movement in a stationary population of an adult. Just as in most stable adult tissues the replicative machinery is switched off or heavily damped down, but is activated by wounding, a process that takes many hours; so it appears that the locomotory machinery is in some way switched off, and after wounding, it likewise may not become available for many hours. This awakening of the ability to move, which I have called mobilisation [19], little though we know about it, has to be kept in mind when considering the stationary state of cells in the organism. The replicative and the migratory state tend to go together, and I suggest that it is only when cells are in this combined state that contact inhibition is relevant to control of their movement.

So much for the non-malignant cell. I now turn to consider the malignant cell in relation to the control of movement by contact inhibition. I am presuming that at least a substantial part of the invasion of surrounding normal tissues from a malignant focus is brought about by the individual cell movement of the kind that I have been discussing for normal cells. It seems to me quite unrealistic to ascribe it all to growth pressure, though it would be rash at present to exclude a contribution from growth pressure. The mobilised normal cell, I have suggested, is brought to a stop by collision with other normal cells; it appears that the malignant cell is not.

In culture, tumorigenic cells often give the impression of a deviation from normal in their pattern of movement. This is especially true of cells transformed *in vitro*, usually by tumor viruses, when the normal precursors are available for immediate comparison. Colonies of such cells in a normal monolayer are very commonly heaped up into a disorderly multilayer [20], which is often thought of as being partly due to cell movement unrestricted by mutual contact inhibition. It should be added that the correlation of this type of colony with the ability to form tumors when implanted into

an animal is by no means perfect; but neither has the analysis of just what behaviour underlies the production of this type of colony as yet been carried out. If the various steps in our hypothesis about the control of cell movement through contact inhibition are valid, we should expect to find that the cells of an invasive tumor were deficient in contact inhibition by normal cells. This has been duly found for a few sarcomas, but I do not think the analysis is fully satisfactory for more than one, the highly anaplastic long-transplanted S180 [21]. The cells of this sarcoma show no sign of contact paralysis on collision with normal fibroblasts, the dorsal surfaces of which they move across. S180 is however highly susceptible to blockage by a non-adhesive surface. We still await an extensive analysis of the locomotory behaviour of an adequate variety of malignant cells.

## REPLICATION CYCLE

The problem of control of the replication cycle in the adult is somewhat similar to that of the control of movement. From the point of view of replication, as of movement, there are large numbers of relatively inactive cells, which can however become active under suitable conditions, e.g. as a result of wounding. As with movement, analysis of tissue cultures has suggested the existence of a control system, involving a simple kind of feed-back, which operates through cell contact. The proposal of a contact inhibition of replication, though it initially arose I think by mistake, acquired real force as a result of Todaro and Green's work with their famous cell-line 3T3 [22, 23], and then of Eagle's work with human diploid fibroblasts [24]. At a density which brings the cells of a monolayer culture of 3T3 into a good deal of mutual contact there is a striking falling off of growth rate. The inhibition could not be transmitted to a less dense culture by means of medium taken from an inhibited culture. This has recently been nicely demonstrated by Schutz and Mora [25], who grew sparse and dense cultures on the two sides of a thin Millipore filter, and found no influence from one to the other. Hence the inhibition seemed to depend on the amount of contact between the cells, and provided a theoretically admirable feed-back system for co-operating with the theoretically admirable feed-back system due to contact inhibition of movement. The latter would ensure that vacant space is filled by redistributing the population so that density becomes even, whatever its absolute level; while the mechanism of contact inhibition of replication can then bring the density up to the standard level.

The experimental results and the ideas centering round the contact inhibition of replication have unfortunately become a great deal more complicated than those around the contact inhibition of movement, probably because more than one group is working on the replication side. There has emerged a replication-promoting substance in the medium that is consumed by the cells [2, 26], and a replication-inhibiting substance in the medium that is produced by the cells [27]. But the original contact idea is not superseded. The closeness of packing of the cells must it seems still affect the intake of the promoter, or the sensitivity to the inhibitor. What however is still not clear is whether the undoubted effect of proximity of the cells really depends on cell contact, that is to say, on an approach sufficiently close to produce adhesion. It could still be that separations measured in microns are close enough for inhibition, and that a substance diffusible extracellularly, but only over an appropriately short range, is the basis of the proximity effect [28].

Consideration of the application of this proximity inhibition to growth control in the embryo produces a slight uneasiness, because one's impression is that the well-known decline of growth rate in the embryo accompanies a diminishing proximity of the cells; but this needs some numerical estimation. Sensitivities may well be changing in the embryo, in any case. In the mature animal wound-healing is a situation where the mechanism, like the contact inhibition of movement, could certainly work. One is reminded here of the theory of locally acting chalones, diffusing specific inhibitors of mitosis, for which experimental evidence has been produced in the case of epidermis [29] and lens [30]. It must however be noted that these chalones have not been shown to inhibit cells earlier than G2, while proximity inhibition except in one case [31] prevents DNA synthesis. Of course in the whole organism one is not confined to local interactions; it seems highly probable that there are a number of instances of specific growth controls acting through feed-back at a systemic level [32]; this means there is a kind of density dependence, but it is the amount of a tissue in relation to the amount of the whole organism that is the operative density. Cultures of a kind that might be expected to demonstrate this sort of control have not been done.

But while the precise relevance of what has been discovered *in vitro* about replication control to what goes on *in vivo* is uncertain, there is in this matter of proximity inhibition of growth a highly suggestive indication from malignant cells, because they tend to lack it. Of course tumours are not growing without restraint [33]. In fact they seem to show the same kind of temporal growth pattern as a perfectly normal embryonic organ. But whatever the growth rate limiting factors are in tumours, they must operate differently from those of normal tissues. And the evidence from tissue culture is that malignant cells, of a considerable variety of kinds, do not respond to proximity inhibition of replication with the same intensity or at the same level as do normal cells [28]. This evidence is better than the corresponding evidence about contact inhibition of movement. The correlation of defective proximity inhibition of replication in culture with tumorigenicity *in vivo* is not, overall, perfect. But within a particularly well-controlled system, Aaronson and Todaro [34] have recently shown a highly convincing correlation.

There are many interesting complications of replication inhibition in the interactions of various kinds of cell with each other [28, 35, 36], as there are incidentally in the case of movement inhibition, but these I shall have to neglect.

Contact control of movement and of replication have therefore, as a result of tissue culture studies, been put on the agenda for further investigation in relation to malignancy. A special role of the cell surface in the controls of movement and growth is suggested; and a further step in the argument, though not a logically necessary one, takes us to the hypothesis that a surface defect is the basis of the different behaviour of normal and malignant cells. There will be evidence bearing on this from other directions later in the conference.

## SUMMARY

*Control mechanisms for cell movement and replication which have been detected in tissue culture and which seem to depend on mutual contact of the cells are discussed. Contact inhibition of movement amongst fibroblasts seems to depend on a contractile response that inhibits locomotion; but some inhibitions may depend on inadequate adhesion between the contacting cells. Contact inhibition of mitosis has a more obscure mechanism. There is suggestive evidence, particularly for the inhibition of mitosis, that both kinds of control may be defective in malignant cells.*

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# The Cell Surface in Immune Response*

EVA KLEIN†
*Department of Tumour Biology, Karolinska Institutet, Stockholm 60, Sweden*

AT THE present state of our knowledge only those antigens which are expressed at the cell surface are responsible for a host response leading to damage and elimination of the cell. Destruction of the neoplastic cell or inhibition of its multiplication are processes where tumor specific antigens may find practical use. The occurrence of tumor specific surface antigens in experimental systems is the rule rather than the exception [1–3]. Their existence indicates a change in the structure of the cell membrane. As growth controlling instructions are probably transmitted to the effector cell organelles by receptors with a cell surface component, a profound change in the normal surface may lead to a deficient responsiveness to controlling forces and can have a direct relationship to the neoplastic transformation.

## TUMOR SPECIFIC TRANSPLANTATION ANTIGENS

Much of the knowledge concerning the host–cell relationship of antigenic cells comes from experimentation with histocompatibility antigens which are also localized on the surface. No property unique to the tumor specific surface antigens has yet been seen with regard to immunological recognition and rejection mechanisms. Strength of the antigenicity varies widely when different systems are studied, but varying strength — reflected by the capacity of the host to reject a graft of a given size and the time necessary for rejection — also characterizes genetically determined isoantigens. One factor which distinctly influences the immune response to an antigenic tumor is the growth capacity which can lead to an overload of antigenic but highly proliferative cells before the immune effector mechanism can cope with them.

Studies on tumor specific surface antigens in animals very soon revealed a very important finding; tumors induced with chemical carcinogens have individual antigens, thus cross immunization is rare or absent even between tumors of similar morphology and induced in the same host genotype [4–6]. This has been found to be the case even when independently induced primary tumors in the same original host were studied [7, 8]. In contrast, virus induced tumors carry a virus determined surface antigen which is common for all the tumors induced by the same virus even if they are different in morphology; cross immunization is thus feasible [9–16]. We know more about the determination of the antigen and its relation to the oncogenic stimulus in virus induced tumors than in the chemically induced ones. The cell surface antigen in tumors induced by DNA viruses is not identical with the antigen of the mature virion. The cellular antigen is present even when infectious virus production by the transformed cells cannot be demonstrated [2]. As the antigen is specific for the inducing virus its presence and permanence provided the first evidence that the virus left part of its genetic information in the cell after neoplastic transformation. In leukemias induced by RNA viruses the cell surface antigen might be shared with that of the virion [17].

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These viruses mature at the cell membrane by budding and during this process, the virus particles, which are continuously shed by the leukemia cells, receive an outer coat derived from the membrane — this can be seen in the electron microscope.

In experimental systems the strength of antigenicity is usually determined in transplantation tests from the maximum number of tumor cells rejected by preimmunized but accepted by untreated syngeneic hosts. While preimmunization either completely inhibits tumor takes or increases the threshold dose by which the tumor can successfully be transplanted, experimental manipulations known to inhibit immune response such as total body irradiation [5], neonatal thymectomy [18], or treatment with anti-lymphocytic serum [19] reduce the threshold dose, indicating that tumor-isograft rejection is indeed immunological in nature.

The classical experiments demonstrating tumor specific cell surface antigens were all done with transplantation tests. Later attention turned to analysis of different parameters of the immune response and the development of *in vitro* test systems. (This was most important as in the case of human tumors the transplantation test cannot be used.) Thus selecting animal host-tumor systems in which antigenicity was proven by transplantation the role of cellular and humoral immunity was studied. Methodology for these studies was also provided by work on the immune response to histocompatibility antigens in which tumor cells were often used as they have the isoantigens determined genetically by the host in which they originated.

## IMMUNOLOGICAL EFFECTOR MECHANISMS

In the immune response against cell surface antigens, be they genetically determined or tumor specific, both cell and antibody mediated effector mechanisms could be demonstrated. Lymph node cells or macrophages from sensitized animals were found to inhibit the growth of the target cells against which they had been immunized [20, 21, 8]. This occurs both in tissue culture system or after mixture *in vitro* and subsequent inoculation into suitable recipient animals. In experiments with chemical carcinogen induced tumors, the sensitized state of lymphocytes followed the same rules as in transplantation experiments, their inhibitory effect being strictly confined to the particular tumor against which the donor animals were immunized [8, 5]. Antibodies in the serum reacting with cell surface could also be demonstrated in some tumor systems. The method of choice for their detection depends on the cell type studied. Mixed haemadsorption and immunoadherence can be used on cells growing as monolayers [22, 23]. Immune serum in the presence of complement can be cytotoxic [24, 13, 25]. Attachment of antibodies to the cell surface can be visualized in the fluorescence microscope [13, 26, 15]. Usually immunofluorescence was found to be of broader applicability than cytotoxicity, because the threshold effects of antigen concentration on the cell surface determines whether the cell will be killed by antibodies [27, 28]. Thus even when cytotoxic reaction in a given cell-serum system does not take place humoral antibodies can still be present and be demonstrated by immunofluorescence.

It was very satisfactory when the sensitized state of lymphocytes and the presence of humoral antibodies could be demonstrated in hosts which rejected tumor grafts, or were immunized with non-living antigen containing material. However, in many cases the signs of an active immune response can be detected in hosts with growing tumors; seemingly the immune response has no effect.

Available information from histoincompatible host-tumor systems offers some explanation for this situation. It is known that the presence of humoral antibodies — present either through active or passive immunization — might facilitate the acceptance of a histoincompatible graft. The phenomenon is called enhancement [29–31]. At present it is believed that *in vivo* most often sensitized cells are responsible for the actual damage to the antigenic target cells. Certain type of target cells — lymphocytes, leukemia cells — might be destroyed solely by circulating antibodies *in vivo*, but it is possible that even in such cases immunologically active cells co-operate in achieving this effect. Although this field of investigation is extremely active now, we still do not know the exact mechanism of the immunological damage [32]. In *in vitro* experiments it was demonstrated that when cell surface antigens are coated with specific antibodies the destructive effect of sensitized lymphocytes can be prevented. The interpretation was that if the antibodies do not kill the cells, they may block the antigen sites and by that these are inaccessible to the lymphocytes. Similar interaction between antibodies and sensitized cells might even occur *in vivo,* although the mechanism of enhancement is certainly more complex [30].

During changes in the host–tumor relation-

ship — a dynamic situation involving concomitant development of humoral and cell mediated response and growth of the tumor — even a temporary enhancing effect by antibodies can lead to unlimited growth because it can allow the tumor to reach a critical size and antigen mass which cannot be controlled by the host's immune response.

Tumor cells vary in their sensitivity to immunological attack. As mentioned above an important role is played by the density of antigen sites at the cell surface. This is due at least in part to the mechanism whereby the complement components are attached and activated. Similar conditions might influence the efficiency of cell mediated immune attack also.

For an effective immune response, leading to rejection of antigenic cells several conditions have thus to be fulfilled. Unfortunately, even if experimentation is vast in this area no measures are known whereby the interplay of the factors can be influenced to reach the desired effect. Consequently we have to bear in mind that the behaviour of tumors under immunological attack may not always be predicted.

It is probable that during carcinogenesis a good number of antigenic cancer cells are eliminated due to a satisfactory sequence of immunological recognition and response. This would imply the release of antigen sufficient for the efferent pathway, recognition before the clone develops to an uncontrollable size, a prompt afferent pathway with a sensitized population of immunocompetent cells and no enhancing effect by antibodies. One experimental series demonstrating the importance of the time factor is provided by Takeda *et al.* [33] in their attempts to demonstrate immunization against tumor metastases in the autochthonous host. Artificial metastases were implanted from methylcholanthrene sarcomas in rats and ligation and release of the primary tumor provided the immunological stimulus. The earlier the time of immunization in relation to the implantation of metastases the more marked was the suppression of the growth of the metastasized tumor.

## STUDIES ON HUMAN TUMORS

In human tumors the main task is still to demonstrate the existence of tumor specific surface antigens. Very convincing evidence for their existence comes from autotransplantation of tumors [34]. Tumor cell suspensions from patients with advanced malignant disease were autotransplanted in different doses to the thigh. The fact that only a large number of cells ($10^8$) took in all cases and that 50% takes were also achieved with $10^6$ cells indicate that the smaller grafts could be successfully rejected. When such inocula were admixed with white blood cells from the patients a decrease in the takes was obtained indicating an inhibitory or toxic effect of all or a part of the white cells [35]. Though this approach seems to be well motivated because of the importance of the demonstration of autologous tumor cell rejection being the most straightforward sign of efficient host response, such investigations are obviously difficult to carry out. The demonstration of antibodies which react with tumor cells and the specificity pattern of such reactivity concerning groups of tumors, the demonstration of lymphocytes capable of damaging tumor cells *in vitro* are the approaches most intensively pursued and evidence is accumulating which lists several tumor types (nasopharyngeal carcinoma, melanoma, neuroblastoma, osteosarcoma) in which such reactions can be found [36–39]. The tumor on which much of the effort is concentrated now in different laboratories using different techniques is the Burkitt lymphoma. This interest was motivated by the finding that all tumors induced in animals by the same virus were shown to have common virally determined cell surface antigens. The geographical distribution of the Burkitt lymphoma was specially suggestive of an infectious etiology. Moreover it was found that response to chemotherapy in these patients is better than in other types of lymphoid malignancies suggesting the cooperation of a host response [40–42]. Indeed in the majority of culture lines derived from Burkitt lymphoma, herpes like virus (EBV) was detected with electron microscopy [43–45]. Efforts to identify the EB virus with other known members of the herpes group gave consistently negative results and it was concluded that the agent is a previously unknown virus of the herpes group.

Serological studies searching for antibodies were conducted on the following lines:

A. Reactivity of serum with intracellular antigens: (1) immunofluorescence on fixed cells from EBV-carrying lymphoblastoid cell lines [46]; (2) complement fixation [47, 48]; (3) precipitin reaction [49] with an antigen preparation extracted from such cells.

B. Reactivity with cell surface antigens: (1) immunofluorescence on living cells [50]; (2) influence on the growth of cultured Burkitt cells [51].

A large number of human sera were found to react with fixed lymphoblastoid culture cells. The reaction visualized by fluorescence was intracellular and occurred in a small proportion of cells. The conclusion that the antigen involved in this reaction is the EB virus was based on the following facts:

1. Burkitt lines free of detectable virus particles in the electron microscope contained no reactive cells.
2. The percentage of virus carrying cells in a cell line determined in the electron microscope corresponded to the percentage of serologically reactive cells.
3. Usually upon serial cultivation the virus is lost from the cultures; when the virus is not detected the proportion of cells which react positively in immunofluorescence decreases to 0·1% or less.
4. A DNA inhibitor capable of inhibiting the multiplication of herpes simplex virus also reduced the number of reactive cells.
5. A direct study demonstrating immunofluorescence and virus particles visualized in the electron microscope was successfully made on the same cells [52].

It was also found that sera from Burkitt patients contain cell membrane-reactive antibodies. This can be demonstrated by exposing living cells to fluorescein-conjugated sera; the antibodies do not penetrate the cells but cell surface reactions can be observed in the fluorescence microscope.

An effort was made to clarify whether the cell membrane antigen is in any way related to or determined by the EB virus. Therefore the presence of EB virus (detected serologically) and the occurrence of cell membrane antigens was surveyed in parallel [53] in a number of culture lines of Burkitt origin and it was found that cells of those lines show surface reactivity which harbor the EBV virus. When virus was no longer present in the cell line, membrane reactivity also disappeared. It is assumed therefore that cell membrane reactivity is associated with the presence of EBV in the culture. There was however, no constant ratio between the proportions of reactive cells. As a rule approximately ten times more cells show membrane fluorescence than have the EBV antigen. Several recently established cultures revealed significant degrees of membrane fluorescence as soon as sufficient numbers of viable cells became available for the first test after the explantation of the biopsy material. EBV positive cells were rare initially but reached relatively high levels on further maintenance of the cultures. Established lines which contained no or very few EBV positive cells were negative in the membrane fluorescence tests as well.

The existence of membrane reacting but EBV negative cells in a cell population indicated that the specificities of the two antigens responsible for the two reactivities are different [54]. This conclusion was further reinforced by the finding that absorption with intact cells removed the cell surface reacting antibodies without affecting their anti EBV titers to any significant degree.

A most important new development came when the EB virus was shown to be related to at least one form of mononucleosis [55]. The clue was obtained when a laboratory technician who served as the donor of EBV negative control serum developed infectious mononucleosis. Her serum subsequently became highly EBV positive. Preillness sera were available from 24 mononucleosis patients. Sampled one month to five years before the onset of symptoms, they were all negative for EBV antibodies. In the course of the disease anti EBV appeared in all of them and rose to titers between 40 and 640 within a few weeks. In view of the suggestion that infectious mononucleosis is a frequent and often unrecognized infection of childhood the wide occurrence of EBV reacting antibodies in the control population could be explained by this finding. The presence of membrane reacting antibodies in these sera paralleled the anti viral reactivities of the sera. In the course of mononucleosis membrane reactive antibodies appeared, reactivity could be seen both with EBV carrying Burkitt lines and with lines established from IM cases. The membrane reactivity of post-mononucleosis sera was maintained through a long period after the illness.

A survey of sera for the presence of virus reacting antibodies and cell membrane reactive antibodies revealed the following: 82 of 206 sera tested, (40%) showed a high anti EBV titer ($>160$) and high membrane reactivity. Low or negative anti EBV activity ($<80$) and low membrane reactivity were found with 81/206 (39%) of the sera. This means that the two tests gave concordant results with 163/206 (79%) of the sera tested. The remaining 21% of the sera showed important discordancies. These discordant sera provided further evidence that different specificities are involved in the two reactions [56].

Besides the sera of Burkitt patients (with high titers 1:640; 1:1280) and the mononucleosis

patients (comparatively lower titers) another category of sera had high anti EBV titers. These were from patients with nasopharyngeal carcinoma [57].

These findings suggest that the EBV virus participates in the etiology of Burkitt lymphoma and nasopharyngeal cancer. A cautionary note may be introduced due to the fact that virus determined antigens can be made to appear in established neoplastic cells by superinfection with a virus known to be oncogenic but bearing no responsibility for the causation of that particular tumor. Such antigens are similar in all respects to the antigens present on and in those tumor cells that have been induced by the same virus. This phenomenon, called antigenic conversion [2] implies that the passenger virus may change the phenotype of the antigenic cell in the same way as the causative virus does. However, the regularity of the association between the EB virus and the antigens probably determined by it irrespective of the geographical areas from which the patients came might be a significant argument against its passenger role.

One has also to consider the possibility that infectious mononucleosis and Burkitt lymphoma are caused by the same virus, or by two closely related viruses. Until now no difference between the two antigen systems has been detected.

Earlier investigations showed a correlation between the strength of cell surface antibodies in Burkitt patients and the response of the patient to therapy [58]. This was taken as a strong suggestion that the serum reactivity reflects an efficient host response. Further studies are in progress, correlating changes in the antibody titer and the clinical status of the patients [59].

## SUMMARY

*The occurence of tumor specific surface antigens in experimental systems is firmly established and evidence is accumulating for their existence on human tumor cells. Both cell and antibody mediated immune reactions against such antigens can be demonstrated. Sensitized lymphocytes and specific antibodies can also be detected in hosts with actively growing tumors. This ineffective immune response is not completely understood; the phenomenon of immunological enhancement, lack or immunosensitivity of the tumor cells may be contributing factors.*

*Tumors induced with chemical carcinogens have individual antigens while virus induced tumors carry a virus determined surface antigen which is common for all the tumors induced by the same virus.*

*The success of chemotherapy in Burkitt Lymphoma suggests the co-operation of immunological host defense factors. The geographical distribution of the tumor points to a viral etiology. These facts motivated extensive immunological studies which revealed that antibodies reacting with:* (1) *the cell membrane of cultured Burkitt cells,* (2) *the herpes virus harbored by such cells,* (3) *the antigen preparations extracted from such cells are present in sera of Burkitt, infectious mononucleosis and nasopharyngeal carcinoma patients.*

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# The Time Factor in Cancer Chemotherapy*

G. MATHÉ, M. SCHNEIDER and L. SCHWARZENBERG

*Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse*
*14, avenue Paul-Vaillant-Couturier, 94-Villejuif, France*
*Service d'Hématologie de l'Institut Gustave-Roussy et Organisation Européenne de Recherche sur le Traitement du Cancer (O.E.R.T.C.)*

DURING the past twenty years, cancer chemotherapy has mainly been guided by the fear of the appearance of toxic side effects, especially cytopenia in the circulating blood. Furthermore, treatment has generally been confined to continuous administration, that is in small daily doses, and if cytopenia occurred the treatment was either stopped or the dose reduced.

The results in the majority of cancers have been poor. Consideration of present day knowledge of the kinetics of cancer cell growth [1], the immunosuppressive effects of cytostatic drugs [2], the existence of special antigens on cancer cells and the immune reactions against them [3], has led us to ask whether intermittent massive doses of chemotherapy might not be more effective than continuous small doses. Earlier experiments in mice [4] have suggested that such a scheme might be advantageous when using methotrexate or cyclophosphamide. This encouraged us to undertake a systematic study of this problem using leukaemia in mice as a model system [5, 6].

Mice carrying L 1210 leukaemia, transmitted by an isogeneic graft of $10^4$ cells, were treated with 7 drugs currently used in cancer chemotherapy (6-methyl-1-hydrocortisone, cyclophosphamide, vincristine, vinblastine, 6-mercaptopurine, methotrexate and daunomycine). The relative effectiveness of these drugs was compared for one or different total doses according to the following scheme: (1) prolonged administration, that is in small daily doses beginning the day after grafting $\frac{1}{8}$ the $LD_{50}$ at 6 days, or a multiple of this dose, for 6 days, then $\frac{1}{12}$ the $LD_{50}$ or its multiple, on alternate days until the 20th day); (2) the single, early administration of the same total dose, of the day following the graft; (3) or the single, late administration of the same total dose, given 6 days after the graft.

Table 1 shows the total doses of the drugs that have been studied. For all these drugs, with the exception of methotrexate and vinblastine, giving a single large dose early gave better results than this single dose given later or as continuous therapy. However, the single dose of vinblastine given late gave better results in comparison to the same dose given early or continuously. The continuous administration of methotrexate in small doses (8·78 mg/kg) and moderate doses (43·9 mg/kg) gave better results than in a single dose, but when the highest total doses (175·6 mg/kg and 263·4 mg/kg) were used and an injection of folinic acid was given after the methotrexate, then a single, early dose was superior to continuous administration.

Figures 1–4 show most significant examples of reactions to this form of treatment.

These results can be explained, at least

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*Table* 1. *Doses used in the various experiments*

| | $LD_{50}$/6 days (Garattini) (mg/kg) | Total dose used in the basic experiment (mg/kg) | Total doses of the other experiments | |
|---|---|---|---|---|
| | | | Inferior to those of the basic experiment (mg/kg) | Superior (mg/kg) |
| Solumedrol | | 126·2 | | 253·4<br>378·6 |
| Vincristine | 0·945 | 1·435 | | 2·870 |
| Vinblastine | 1·17 | 1·84 | | 5·52 |
| 6-Mercaptopurine | 144 | 228 | | |
| Methotrexate | 5·56 | 8·78 | | 43·9<br>87·8<br>175·6<br>263·4 |
| Cyclophosphamide | 255 | 403·4 | 134·5<br>80·7 | |
| Rubidomycin | 13·8 | 21·85 | 7·28 | 43·70 |

partially, from data on cell kinetics of normal haemopoietic and leukaemic cells [1]: a certain proportion of normal haemopoietic cells are in Go, whilst all L 1210 cells are engaged in cyclic division [5, 7]. In our experiments, we have used drugs whose action is directed against dividing cells or against a particular part of the cycle. Bruce and his associates [1] have shown that for these drugs, due to the reasons given above, the percentage of leukaemic cells killed is much higher than the number of haemopoietic cells killed in relation to the dose. The best results observed in our experiments were better with a single high dose than with repeated smaller doses. It is evident that the final result is dependent on the number of leukaemic cells that are killed and the number of haemopoietic cells that are spared to restore blood production.

In another experiment, we have demonstrated that if the same total dose of cyclophosphamide (403·4 mg/kg) has an identical immunosuppressive action, as indicated by the delayed rejection of an allogeneic skin graft, whether it is given either as a single dose or in smaller repeated doses ($\frac{1}{6}$ of the $LD_{50}$ at 6 days daily, followed by $\frac{1}{12}$ of this dose on alternate days until the 20th day), this phenomenon was not

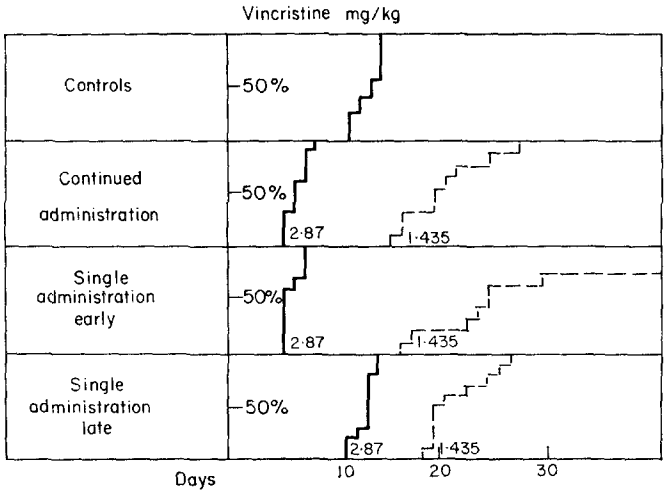


*Fig.* 1. *Cumulative survival curves of (DBA/2×C57Bl/6) F1 mice, carrying* L 1210 *leukaemia, treated with two doses of vincristine, according to various schedules.*
*Twenty-one mice were used as controls.*
*Twenty-nine mice received* 1·435 mg/kg: 9 *as a continuous treatment;* 9 *in a single early dose;* 10 *in a single late dose. Thirty mice received* 2·87 mg/kg: 10 *as a continuous treatment;* 10 *in a single early dose;* 10 *in a single late dose.*

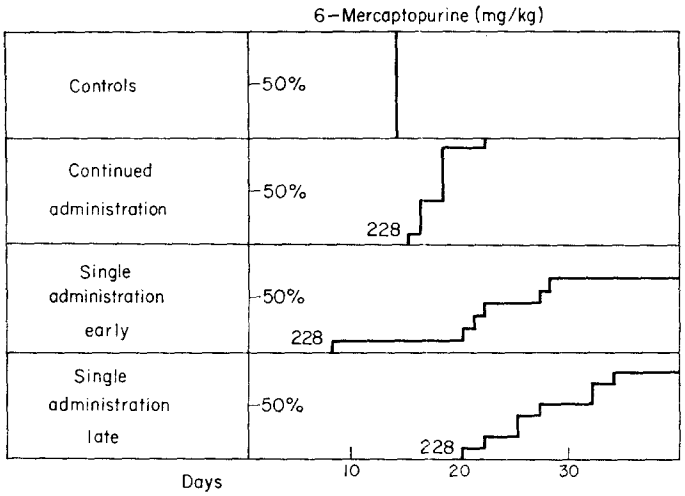


*Fig.* 2. *Cumulative survival curve of animals carrying* L 1210 *leukaemia, treated with the same dose of* 6-*mercaptopurine, according to various schedules.*
*Eight mice were used as controls.*
*Twenty-nine mice received* 288 mg/kg: 10 *as a continuous dose;* 9 *in a single early dose;* 10 *in a single late dose.*

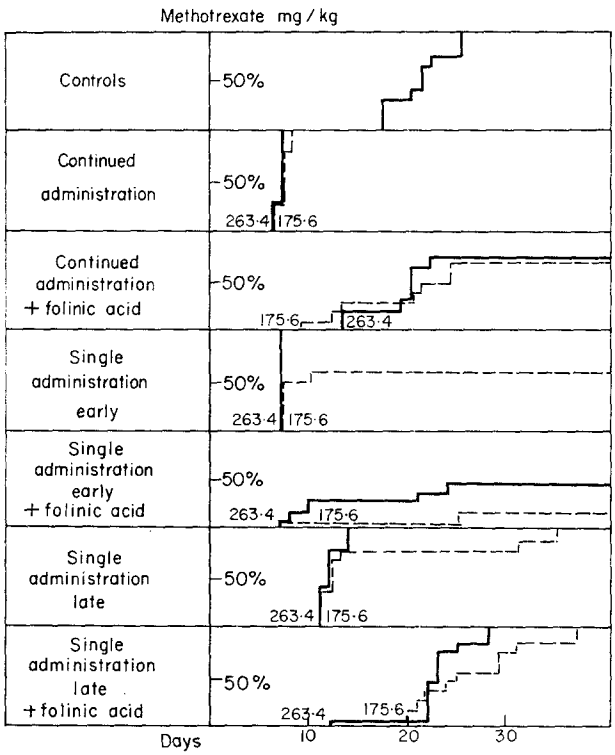


*Fig.* 3. *Cumulative survival of animals carrying* L 1210 *leukaemia, treated with two total doses of methotrexate, according to various schedules. Folinic acid was given at the same dose* 24 *hr after this drug.*
*Eighteen mice were used as controls.*
*Sixty mice received* 175·6 mg/kg: 10 *in continuous treatment+folinic acid;* 10 *in a single early dose;* 10 *in a single early dose+folinic acid;* 10 *in a single late dose;* 10 *in a single late dose+folinic acid.*
*Sixty mice received* 263·4 mg/kg: 10 *as continuous treatment;* 10 *in a single early dose;* 10 *in a single early dose+folinic acid;* 10 *in a single late dose;* 10 *in a single late dose+folinic acid.*

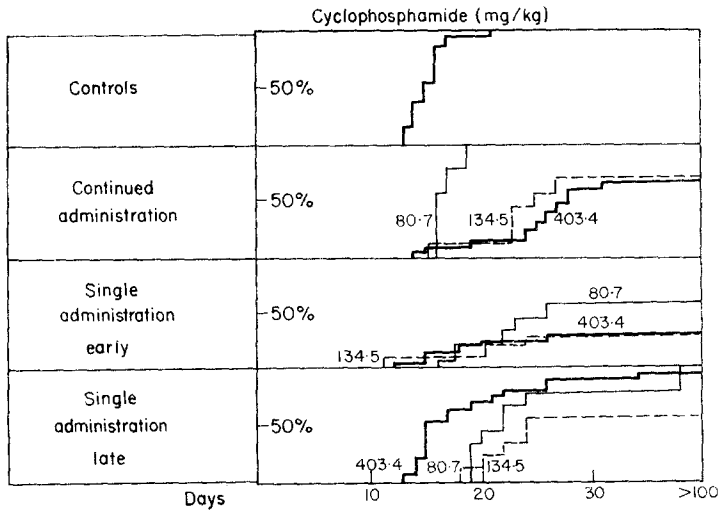


*Fig. 4. Cumulative survival curves of animals carrying* L 1210 *leukaemia, treated with different total doses of cyclophosphamide, according to various schedules.*
*Forty mice were used as controls.*
*Twenty-seven mice received* 80·7 mg/kg: 9 *as continuous treatment; 9 in a single early dose; 9 in a single late dose.*
*Twenty-eight mice received* 134·5 mg/kg: 9 *as continuous treatment;* 10 *in a single early dose; 9 in a single late dose.*
*Sixty mice received* 403·4 mg/kg: 21 *as a continuous treatment;* 20 *in a single early dose;* 19 *in a single late dose.*

observed when methotrexate was used: giving 8·75 mg/kg in a single dose did not retard the rejection of an allogeneic skin graft, whilst the same total dose given in smaller repeated doses ($\frac{1}{16}$ of the $LD_{50}$ at 6 days, daily for 6 days, the $\frac{1}{12}$ of this dose on alternate days until the 20th day) caused significant delay in the grafts rejection (Fig. 5).

These experiments have induced us to replace continuous daily low dose schedules of cycle and phase dependent chemotherapeutic drugs by intermittent high dose therapy. This was done for the following reasons: (1) high intermittent doses seem to be more effective against tumour cells, when they are all dividing, than against haemopoietic stem cells; (2) the single high dose is less immunosuppressive.

Clearly these conclusions are of no value for drugs acting equally well on in cycle and Go cells, such as methyl-bis-β-chloroethymamine [1]. And one knows that, in many spontaneous tumours, especially solid tumours, there are

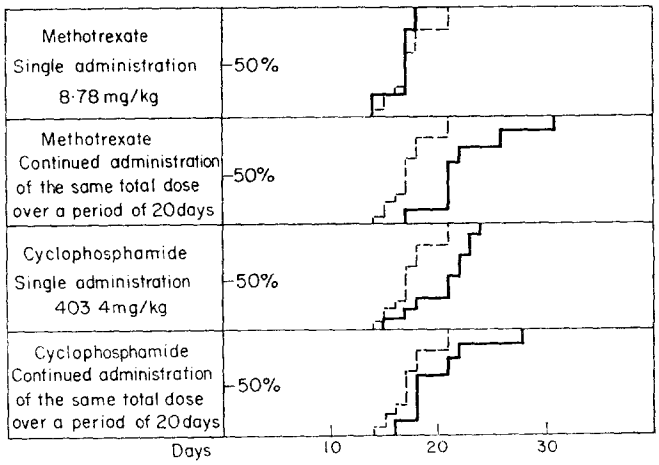


*Fig. 5. Comparison of the cumulative survival of allogeneic skin grafts on mice treated with the same total dose of methotrexate or cyclophosphamide, according to different schedules.*
*14 mice acted as controls.*
*Twelve mice received* 8·78 mg/kg *methotrexate:* 5 *as a single dose,* 24 *hr after the graft;* 7 *as continuous treatment for* 20 *days. Seventeen mice received* 403·4 mg/kg *cyclophosphamide:* 10 *as a single dose;* 7 *as a continuous treatment for* 20 *days.*

cells that are not engaged in cyclic division, specially as the result of the paucity of their blood supply.

In clinical practice, it is impossible to get any precise estimates of the percentage of tumour cells that are engaged in cyclic division. Tubiana and his co-workers [8] have been able to measure the growth fraction in five untreated skin tumours in man. They found values situated between 29–40%. This has indicated that the majority of the cells in human solid tumours will not be engaged in cyclic division at any moment. It is well known that the doubling time of the tumour cannot give precise estimate of the generation time, due to the considerable coincidental cell loss. Nevertheless, we base our choice of schedule for giving chemotherapy upon the doubling time.

When the doubling time (DT) is short (it is known to be 4 days in acute leukaemia), it suggests that the percentage of dividing cells is high; in undifferentiated tumours, this will apply to a major fraction of the cells having the morphological features of blast cells. The kinetics of this type of tumour can be comparable to that of L 1210 leukaemia.

It is reasonable to consider that at any moment in time a high proportion of the tumour blast cells will be in cycle, and that a considerable proportion will be in the S phase. Therefore it seems logical to turn to intermittent use of cycle or phase dependent drugs in the highest possible dosage for patients with tumours of this type. We have used the following protocol for giving methotrexate with the addition of folinic acid to counteract some of its side effects. High doses of methotrexate (75 mg/m2 every 8 hr) for 48 hr, followed by giving folinic acid (25 mg/m2 every 6 hr) for 4 days; this therapy can be repeated weekly. In this way, all the cells that were in the S phase would have been submitted to a minimal concentration of $10^{-6}$ g/ml methotrexate, an *in vitro* concentration that kills all cells.

The clinical results have been outstanding: they are shown in Tables 2 and 3 and in Fig. 6–9, which give examples of the regressions obtained. In a patient suffering from leukaemia remaining resistant to this therapy by doubling the dose a remission was induced in a week (Fig. 10).

When the doubling time is long (it can be several months), it is likely that the percentage of cells in cycle or any phase of the cycle is very small. This applies to differentiated tumours, where morphological examination shows that the majority of the cells to be differentiated have lost their ability to divide, and the number of blast cells is low. We are attempting to destroy these blast cells but, from their rate of production, very few of them appear to be in cycle, which is very different from the situation in undifferentiated tumours.

Theoretically, it is of little value to submit a patient with a slow growing tumour to intensive chemotherapy, which limits the patient's freedom, is not without dangers, and requires hospitalization. It seems advantageous to use an association of several drugs, acting on different phases of the cycle, so as to affect the

*Table 2. Results obtained by methotrexate followed by folinic acid in acute lymphoblastic leukaemia and in acute myeloblastic leukaemia*

| | Visible phase No. | Number of cases | Remissions | | Failures | |
|---|---|---|---|---|---|---|
| | | | Apparently complete | Partial | Partial | Complete |
| Primary acute lymphoblastic leukaemia | 1 | 3 | — | — | — | 3 |
| | 2 | 6 | 3 | — | 1 | 2 |
| | 3 | 4 | 2 | — | — | 2 |
| | 4 | 1 | 1 | — | — | — |
| Total | | 14 | 6 | 0 | 1 | 7 |
| Acute leukaemia syndrome during lymphosarcoma | | 2 | 1 | — | 1 | — |
| Total of the acute lymphoblastic leukaemias | | 16 | 7 | 0 | 2 | 7 |
| Acute myeloblastic leukaemia | 1 | 1 | — | 1 | — | — |
| | 2 | 2 | 1 | — | — | 1 |
| Total | | 3 | 1 | 1 | 0 | 1 |

*Table 3. Results obtained by methotrexate followed by folinic acid in various solid tumours with a short doubling time*

| | Regressions | | Failures | |
|---|---|---|---|---|
| | Apparently complete | Partial | Partial | Complete |
| Breast cancer | 3 | 3 | 3 | 3 |
| Osteosarcoma | | 1 | | 1 |
| Melanosarcoma | 1 | 1 | 1 | |
| Lung cancer | | 1 | | |
| Rectal cancer | | 1 | | |
| Cancer of the nasopharynx | | 1 | | |
| Cancer of the kidney | | | | 1 |
| Testicular choriocarcinoma | | | | 1 |
| Angiosarcoma | | | 1 | |
| Rhabdomyosarcoma | | | | 3 |
| Bronchial carcinoma | 1 | | | 2 |
| Total | 5 | 8 | 5 | 11 |

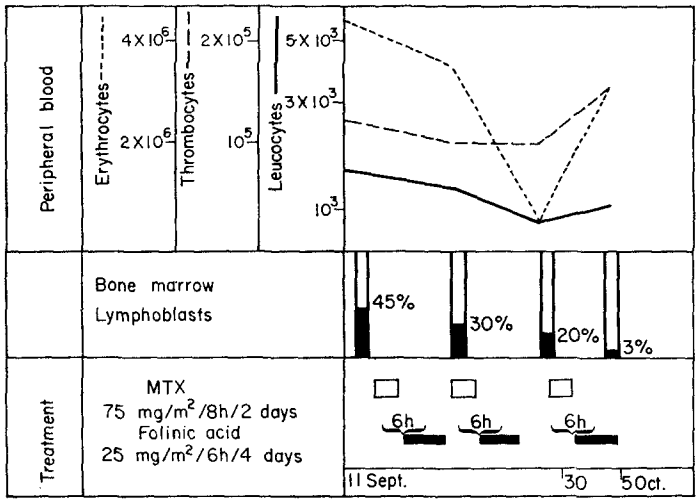


*Fig. 6. Induction of a remission in a patient suffering from acute lymphoblastic leukaemia, with the combination of methotrexate* (75 mg/m² *every* 8 *hr during* 48 *hr*), *followed by folinic acid* (25 mg/m² *every* 6 *hr during* 4 *days*).

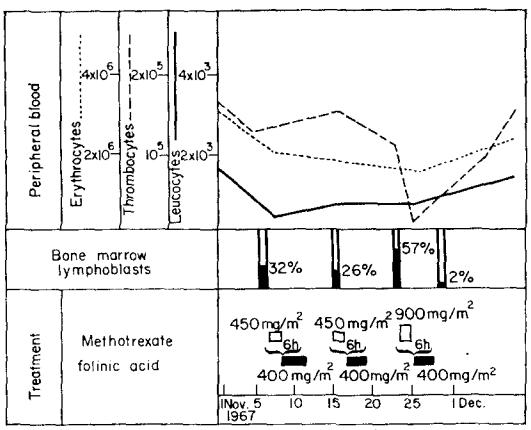


*Fig. 10. Rapid induction of a remission with doubling the dosage of methotrexate-folinic acid in acute lymphoblastic leukaemia. This patient was insensitive to the normal dose shown in Fig. 7.*

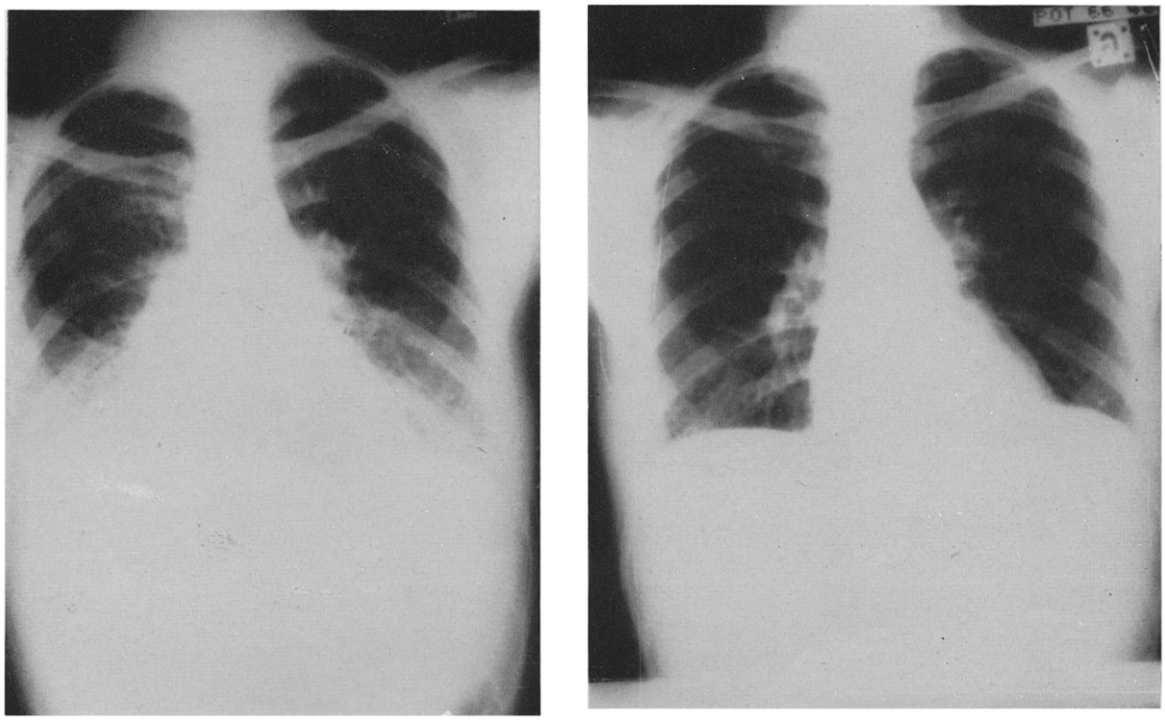

*Fig. 7. Effects of the combination of methotrexate, 75 mg/m2/8 hourly for 48 hr, and folinic acid, 25 mg/m2/6 hourly for 4 days, on a thoracic metastases from breast cancer.*

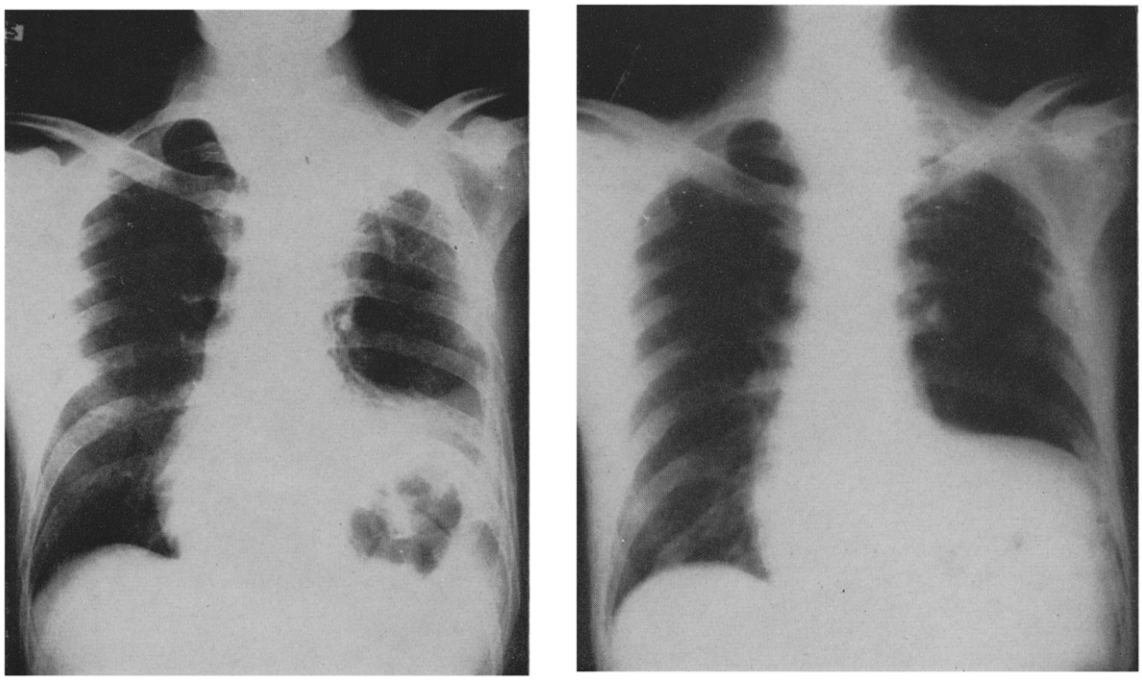

*Fig. 8. Effects of the combination of methotrexate and folinic acid therapy (same dose as in Fig. 6) on a bronchial carcinoma.*

(to face p. 28)

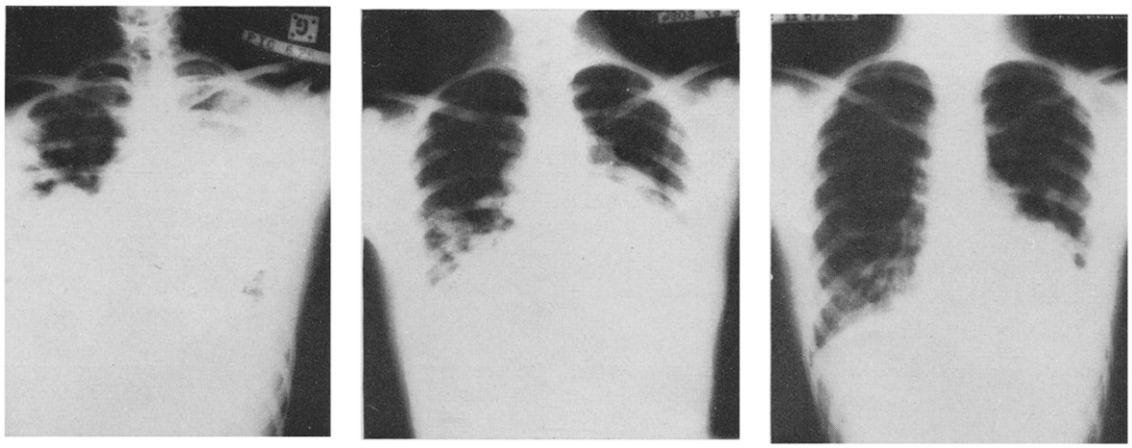

*Fig. 9. Effects of the combination of methotrexate and folinic acid therapy (same dose as in Fig. 7) on a thoracic metastases from osteosarcoma.*

greatest number of cells, whatever be their condition in relation to the cycle at the time the drug is given. These drugs should have different toxicities in order to avoid an accumulative toxic effect.

Table 4 gives different types of intermittent chemotherapy that we use for tumours with long doubling time. The associations are generally given every second week; in practice they are only prescribed when the leucocyte count has returned to normal (Fig. 11).

Chemotherapy of slowly growing tumours, where only few cells are blast cells, will not be accompanied by dramatic results. The chemotherapy should be continued for a long time. Nevertheless it should only by pursued if, after a suitable time interval, a tendency for the tumour to regress has been observed.

The schemes that we have outlined in Table 4 can be followed for several months or years; this is one of their main advantages. The trials that we have made using these schemes have not been comparative with trials of continuous administration. We can only mention the high number of good results obtained (Table 5 gives an example), without accidents,

*Table 4. Example of some associations that can be used in the treatment of tumours with a long doubling time*

| | | |
|---|---|---|
| Digestive tract | 5-Fluorouracil | 1 200 mg/mg2/once |
| | Cyclophosphamide | 300 mg/m2/once |
| | Vinblastine | 6 mg/m2/once |
| Testis | Actinomycin D | 0·4 mg/m2/during 5 days |
| | Methotrexate | 60 mg/m2/once |
| | Cyclophosphamide | 300 mg/m2/once |
| Placental choriocarcinoma | Methotrexate | 60 mg/m2/once |
| | Vinblastine | 6 mg/m2/once |
| Sarcoma of soft tissues | Actinomycin D | 0·4 mg/m2/during 5 days |
| | Vincristine | 2 mg/m2/once |
| Other solid tumours | Methotrexate | 60 mg/m2/once |
| | Vinblastine | 6 mg/m2/once |
| | Cyclophosphamide or | 300 mg/m2/once |
| | 5-Fluorouracil | 1 200 mg/m2/once |
| | Thio T.E.P.A. | 20 mg/m2/once |
| | Vincristine | 1 mg/m2/once |
| | (Intermittent administration, type II) | |

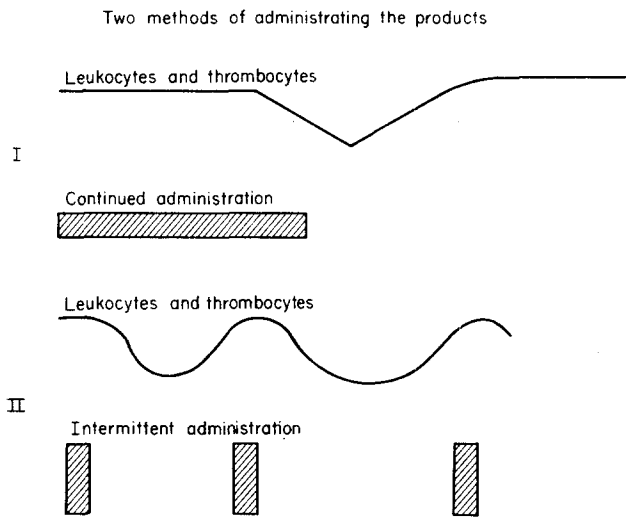


*Fig. 11. Two methods of giving the drugs. I. Drugs acting on in cycle and Go cells: continuous administration in small doses (prolonged); II. Drugs that are cycle or phase dependent: intermittent high dose therapy if the leucocyte count is normal or after it has returned to normal values.*

and with the minimum disturbance for the patient. All these schemes are suitable for outpatients.

Another very important advantage of intermittent chemotherapy is its less immunosuppressive effect, compared to continuous chemotherapy: this is shown in Table 6, which is a summary of a study made in our laboratory by Schneider [9].

*Table* 5. *Results obtained by vinblastine+methotrexate+cyclophosphamide (see Table* 4) *in various solid tumours with a long doubling time*

| | Regressions | | Failures | |
|---|---|---|---|---|
| | Apparently complete | Partial | Partial | Complete |
| Breast cancer | 4 | 12 | | 9 |
| Cancer of the kidney | | | 1 | |
| Osteosarcoma | | | | 1 |
| Cancer of the uterus | | | | 1 |
| Cancer of the bladder | | | | 1 |
| Bronchial carcinoma | 2 | | | 2 |
| Nevocarcinoma | | 3 | | 2 |
| Cancer of the ovary | | 2 | | |
| Cancer of the digestive tract | | | | 1 |
| Sarcoma of the prostate | | 1 | | |
| Cancer of the larynx | | | | 2 |
| Rhabdomyosarcoma | | | | 1 |
| Secondary cancer of the liver | 1 | | | |
| Total | 7 | 18 | 1 | 20 |

*Table* 6. *Comparison of the immunosuppressive effects obtained with continuous and discontinuous chemotherapies in man**

| | Absence of visible immunosuppressive effect | Visible immunosuppressive effect |
|---|---|---|
| Discontinuous chemotherapies | 51/57 (89·4 p. 100) | 6/57 (10·6 p. 100) |
| Continuous chemotherapies | 9/27 (33·3 p. 100) | 18/27 (66·6 p. 100) |

*The patients in whom the delayed hypersensitivity responses was negative before and after chemotherapy and in whom it was impossible to conclude an eventual immunosuppressive effect of the treatment do not appear in this table.

## SUMMARY

*The recent information on tumour cell and haemopoietic stem cell kinetics has led us to replace, for phase or cycle dependent chemotherapeutic agents, continuous doses by large intermittent doses. An experimental study has shown that, for most products, the superiority of the antitumoral effect is more important with a single administration of the same total dose then when given over a period of* 20 *days. Some drugs show that a single dose has a less immunosuppressive effect than the prolonged low dose administration; this has been confirmed clinically. Clinical studies have enabled drug selection to be rationalized according to the tumour doubling time and histological type. The results of intermittent high dose therapy are encouraging and their tolerance is remarkably good.*

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# The Problem of Anaemia in the Acute Leukaemias

## Kinetic Study*

F. GAVOSTO, V. GABUTTI, P. MASERA and A. PILERI
*Haematology Laboratory, General Medical Clinic, University of Turin, Italy*

LEUKAEMIAS are proliferative (chronic form) and maturative (acute form) disorders of individual haematopoietic cell lines. In addition, however, both forms are accompanied by secondary involvement of other cell lines which appear at first to be unimpaired by the leukaemic process. Such involvement is relatively early in acute and relatively late in chronic leukaemia and histogenetic and kinetic studies of the cell population must of necessity be extended to include apparently undiseased lines.

Anaemia is the most common complication of the disease and it has long been considered, at least in general terms, as a secondary lesion due to invasion and overpopulation of the marrow by leukaemic cells. Recent experimental evidence, on the other hand, hints at primary compromise of the stem cell compartment replenishing the erythroblastic series: for example, the same chromosome abnormality has been shown in the myeloid and erythroid lines of patients suffering from chronic myeloid [1–3] or acute leukaemia [4]. The modern view of an omnipotent stem cell capable of producing either erythroid or myeloid progeny suggests that, in leukaemia, there could be a kind of competition at the stem cell level: the predominant demand for blood cells typical of the leukaemia form in any particular case could, in fact, prevent the modulation of the totipotent cell to an erythropoietic committed stem cell. Alternatively, anaemia may be an expression of decreased proliferative and/or maturative activity on the part of the erythroid cells or of ineffective erythropoiesis as in sideroblastic anaemia [5], erythremic myelosis [5] and pernicious anaemia [6]. Earlier *in vivo* studies with $^3$H-thymidine have shown that the residual erythropoietic cells proliferate in a manner very similar to that of normal erythroid precursors, though it has not yet been possible to determine cell fluxes and transit times [7].

We have made a closer examination of this problem by carrying out an *in vivo* erythrokinetics study in 3 previously untreated cases of acute myeloblastic leukaemia (AML) accompanied by a notable degree of anaemia. Marrow blood smears were obtained at different times after the administration of a single dose of $^3$H-thymidine (0·25 μCi/g of body weight-specific activity 9c/mM) and prepared in accordance with conventional autoradiographic techniques [8]. At least 1000 cells were counted at each time interval.

Figure 1 presents our observed labelling index (LI) values for the following erythroid types: proerythroblasts and very large basophil erythroblasts ($E_1$); medium and small basophil erythroblasts ($E_2$); early polychromatophil erythroblasts ($E_3$); late polychromatophil and orthochromatic erythroblasts ($E_4$), i.e. the 100% non-proliferative pool. One hour after labelling these values are very similar to those reported by us for the normal subject [9]; the same can also be said for the relative compartment size values or, in other words, the maturation rates (Tables 1–3).

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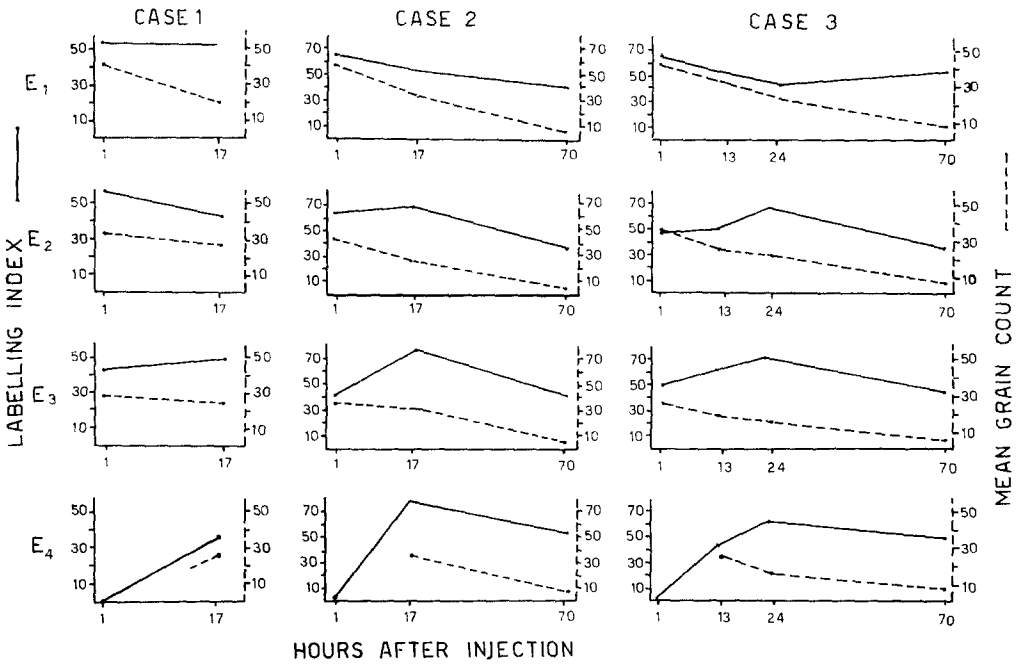


*Fig.* 1. *Labelling indices and mean grain count of different erythroid cells after* $^3$*H-thymidine injection in three acute leukaemia cases.*

*Table* 1. *Kinetic characteristics of erythroblasts in case* 1 *(estimated S period:* 13 *hr)*

| | $E_1$ | $E_2$ | $E_3$ | $E_4$ |
|---|---|---|---|---|
| Relative No. | 100 | 258 | 344 | 732 |
| Labelling index | 53 | 54 | 42 | |
| Halving time of $E_1$ for grain count, hr | ~16 | | | |
| Minimum transit time, hr | | 17·3 | 13·1 | |
| Transit time, hr | 24·5 | | | |
| Maximum transit time, hr | | 63·3 | 23 | |
| Birth rate | 4 | 10·8 | 11·3 | |
| Influx | | 4 | 14·8 | 14·8* |
| Efflux | 4 | 14·8 | 26·1 | |

*Based on 2·05% per hr replacement of unlabelled $E_4$ by labelled $E_3$

*Table* 2. *Kinetic characteristics of erythroblasts in case* 2 *(estimated S period:* 13 *hr)*

| | $E_1$ | $E_2$ | $E_3$ | $E_4$ |
|---|---|---|---|---|
| Relative No. | 100 | 339 | 708 | 626 |
| Labelling index | 65 | 64 | 41·1 | |
| Halving time of $E_1$ for grain count, hr | ~25 | | | |
| Minimum transit time, hr | | 15·6 | 16·1 | |
| Transit time, hr | 20 | | | |
| Maximum transit time, hr | | 67·8 | 32·6 | |
| Birth rate | 5 | 16·7 | 22·4 | |
| Influx | | 5 | 21·7 | 27·4* |
| Efflux | 5 | 21·7 | 44·1 | |

*Based on 4·38% per hr replacement of unlabelled $E_4$ by labelled $E_3$

*Table 3. Kinetic characteristics of erythroblasts in case 3 (estimated S period: 13 hr)*

| | $E_1$ | $E_2$ | $E_3$ | $E_4$ |
|---|---|---|---|---|
| Relative No. | 100 | 417 | 664 | 770 |
| Labelling index | 65 | 49·2 | 50·4 | |
| Halving time of $E_1$ for grain count, hr | ~35 | | | |
| Minimum transit time, hr | | 20·1 | 14·2 | |
| Transit time, hr | 20 | | | |
| Maximum transit time, hr | | 83·4 | 31·9 | |
| Birth rate | 5 | 15·7 | 25·7 | |
| Influx | | 5 | 20·7 | 22·8* |
| Efflux | 5 | 20·7 | 46·4 | |

*Based on 2·96% per hr replacement of unlabelled $E_4$ by labelled $E_3$.

The highest 1 hr post-labelling LI values were observed in the $E_1$ compartment and may be contrasted with the constantly nil values presented by $E_4$. Values remained high till the 70th hr and were accompanied by a clear-cut gradual mean grain count (MGC) fall (Fig. 1). The nil values of the $E_4$ group were gradually replaced in all three cases as follows: 34·6% at the 17th hr (case 1); 73% and 55·9% at the 17th and 70th hr (case 2); 43%, 63% and 49% at the 13th, 24th and 70th hr respectively (case 3). Figure 1 also shows that MGC values were virtually the same for $E_3$ at the 1st hr and for $E_4$ at the 13th–17th hr. Grain distribution studies in case 3 showed that about 17% of the $E_3$ compartment presented more than 30 grains 1 hr after labelling (Fig. 2), whereas about 10% of the cells in the $E_4$ compartment still had more than 30 grains at the 24th hr.

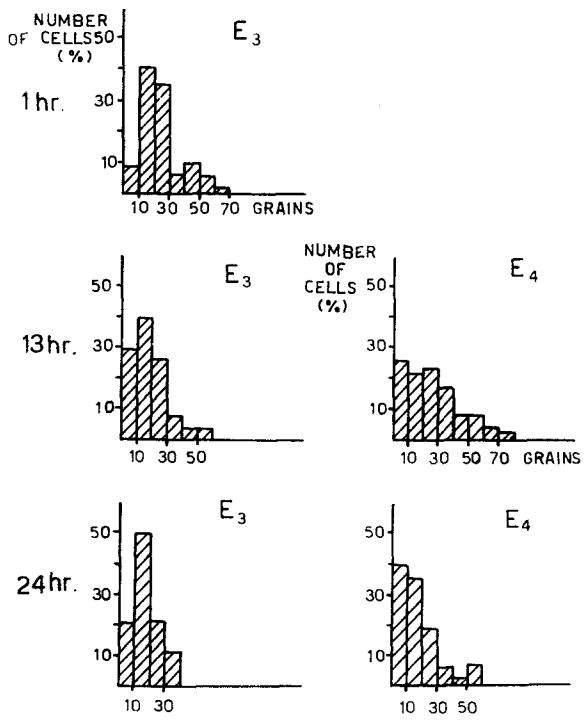


*Fig. 2. Histograms of the grain count distribution for early and late polychromatophil erythroblasts at different time from $^3$H-thymidine injection.*

Inter-compartment cell flux and compartment transit time were then determined. Evaluation of the *S* phase on the basis of the labelled mitoses curves was prevented by the limited number of samples obtained from the 3 cases in our series. We therefore estimated the flux rates through the erythropoietic compartment by assuming two possible *S* phase values, namely: (1) 13 hr, following Stryckmans *et al.* [10] (value observed for normal erythroblasts); (2) 16 hr, following our own finding in a case of acute myeloblastic leukaemia, in which labelled and non-labelled marrow blast mitoses were counted after a single thymidine pulse [11]. Compartment transit times are equal to those of normal cells in the case of the $E_1$ compartment and slightly longer for both the $E_2$ and $E_3$ compartments [12] (Tables 1–3).

Cell flux rates were determined on the 1 hr post-labelling sample, plus the 17th hr (case 1), 17th hr (case 2) and 13th hr (case 3) samples respectively. The $E_4$ replacement rate was calculated by reference to the influx of labelled cells into the non-labelled $E_4$ compartment, using the method reported by Patt in the dog [13] and Cronkite in man [14]. Tables 1–3 show the erythropoietic cell reproduction rates for our three cases, assuming an *S* value of 13 hr. It is clear that the $K_{out}$ of the proliferating into the non-proliferating compartment is well below the sum of the individual compartment birth rates: ineffective red cell production at the $E_4$ level is indicated, whether the *S* phase be 13 or 16 hr.

## DISCUSSION

Ineffective red cell production in AML is amply demonstrated by the kinetics data obtained in our 3 untreated cases: birth rates

were, in fact, less than flux rates. Comparison of the behaviour of the mean grain count and grain distribution values observed in case 3 provides further data for the assessment of the efficiency of erythropoiesis. At the end of the first hour, the $E_1$ compartment already displayed more than 17% of cells with over 30 grains, whereas the $E_4$ compartment showed no signs of labelling. At the 13th and 24th hr, while the mean grain count value had fallen considerably in the case of the $E_1$ compartment, the $E_4$ grain distribution pattern showed no meaningful change with respect to the percent of cells with more than 30 grains. This finding is clearly inconsistent with the possibility that the early polychromatophil cells proceed by direct division into late polychromatophil cells, but is in keeping with the hypothesis of a divisionless maturation.

Transit times fall within a range of 52 to 122 hr (mean: about 80 hr) (Tables 1–3). Since our data (cases 2 and 3) show that the 1st hr $E_1$ compartment mean grain count was 58 and 43 respectively, whereas that of the $E_3$ compartment at the 70th hr was 8 in both cases, we may assume that 3–4 divisions were necessary for the 1 hr-labelled $E_1$ cohort to mature to the status of $E_3$ cells by the 70th hr (Fig. 1).

An outstanding feature of the $E_1$ compartment kinetics is the very slight fall of LI values during these assumed 3–4 divisions, accompanied by a clear decrease of MGC values to about an 1/8. The observation of stationary LI combined with falling MGC values lends support to the postulation of this number of divisions (bearing in mind that the last MGC value may be over-estimated on account of the scarcity of grains). It also indicates: (1) that, after each division, about 50% of the daughter cells are still $E_1$ cells, and (2) that the insertion (if any) of a stem cell feeder compartment must be very slow, with the result that the leading feature of such a compartment is probably a slow reproductive cycle and/or a large number of out of cycle cells.

In more general terms, therefore, it may be suggested that fewer red cell mitoses than normal take place during transit into the proliferating compartment in leukaemia. The possibility that one step in the differentiation process may be omitted has been also investigated by Stohlman [15] in terms of cell response to erythropoietic stimulus: in addition to its effect on the stem cell, a strong stimulus would block haemoglobin synthesis. Critical cytoplasmatic concentration would trigger a feedback process and the skipping of one cell division. It is, in fact, known that above-normal erythropoietin levels are a common part of the acute leukaemia picture.

The conclusion we can draw from our data, then, is one of abnormal erythroblast kinetics in acute myeloblastic leukaemia, resulting in signs of ineffective red cell production. This, in turn, may be accepted as a cause of the anaemia that accompanies the principal disease, though it is not suggested that other causes are thus excluded.

Mention may be made of the mechanisms that may be responsible for ineffective erythropoiesis in AML. The decreased erythroblast pool of acute leukaemia may be partly attributed to the existence of a common stem cell for both the myeloid and erythroid series, such cell being committed in the myeloid sense as a means of compensating a non-absolutely functioning myeloid-granulocyte production. This would decrease the number of stem cells available for erythroblastic differentiation: the differentiative block typical of the leukaemic process may be presumed to occur in the myeloid-committed stem cell compartment. A competition of this kind has been observed by Hellman and Grate in the normal mouse stem cell compartment as a response to various toxins [16]. Killmann has advanced the view that, in the acute forms of the disease, even erythropoietically differentiated cells are leukaemic in the sense that they are derived from the same stem cells as the blasts, but are not thereby handicapped to any great degree in following their normal differentiative path [17]. This view is primarily based on the direct observation of very similar erythroblast and blast cell chromosome abnormalities [4], from which it may reasonably be deduced that decreased efficiency in the production of red cells is a direct consequence of genome alteration. Other factors dependent on the reciprocal relationship between the erythropoietic cell and the leukaemic blast may be involved, however. Factors of this type may be represented by possible metabolite deficiencies (sometimes suggested by the macrocytic and megaloblastoid appearance of the erythroblasts themselves), or be directly associated with events of an immunological type or with the release of substances (e.g. polypeptides) inhibiting physiological growth and differentiation in the normal host cell [18]. This means that a different approach must be made to the classic view that anaemia in acute leukaemia is primarily the result of invasion of the marrow by leukaemic blasts, with the consequent displacement of the normal red cell precursors. It does not

necessarily follow that hyperplasia of a leukaemia-modulated haematopoietic cell line must result in a drastic reduction of the precursor pool of another line (in the present case, the erythropoietic line). The fact that disturbed red cell production may itself be an expression of erythroid precursor abnormalities at the stem cell level may be fully sufficient as an explanation of the anaemia so commonly observed in the acute leukaemia patient.

## SUMMARY

In vivo *pulse-labelling with $^3$H-thymidine was employed to obtain red cell kinetics data in 3 untreated cases of acute myeloblastic leukaemia (AML). The* $K_{out}$ *of the proliferating into the non-proliferating compartment was below the sum of the individual compartment birth rates in all 3 cases: this sign of ineffective red cell erythropoiesis was equally evident whether an S phase of 13 or 16 hr was assumed. Grain distribution values provided evidence in favour of a divisionless maturation of erythroblasts. A fall in the number of stem cells available for erythroblastic differentiation and ineffective erythropoiesis are held to be the principal mechanisms responsible for anaemia in AML.*

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# Etude Comparative des Epithéliomas Médullaires du Corps Thyroïde avec et sans Stroma Amyloïde

M. TUBIANA, C. PARMENTIER, R. GERARD-MARCHANT, J. LACOUR, G. MILHAUD, B. BOK, et J. WEILER

*Institut Gustave-Roussy, 94 Villejuif, France*

La notion d'épithélioma médullaire est relativement ancienne, mais c'est seulement en 1959 que Hazard, Hawk et Crile [1] décrivent sous le terme d'épithélioma médullaire à stroma amyloïde une nouvelle entité anatomo-clinique de cancer thyroïdien, dont ils rapportent 21 observations. Ses caractères essentiels sont: structure cytologique peu différenciée, architecture solide, présence de substance amyloïde dans le stroma, fréquence des métastases ganglionnaires cervicales, malignité intermédiaire entre celle du cancer vésiculaire et celle de l'épithélioma papillaire du corps thyroïde.

De nombreuses publications [2–10] précisent bientôt la symptomatologie clinique, ainsi que la possibilité d'associations morbides diverses [10–17]. Elles soulignent d'autre part le caractère familial de certaines observations [2, 8, 9, 10, 12, 18, 19]. L'étude récente d'anomalies chromosomiques, chez certains malades et différents membres de la même famille, milite en faveur de la nature héréditaire de l'affection [20]. Enfin, la mise en évidence de thyrocalcitonine dans le sang des patients et dans la tumeur elle-même vient de fournir un support biochimique à cette entité morphologique [21–24].

Or, la littérature, très riche quant aux épithéliomas médullaires à stroma amyloïde, est en revanche d'une discrétion extrême en ce qui concerne les cancers médullaires sans stroma amyloïde. De sorte qu'actuellement, une certaine confusion paraît possible, et d'aucuns pourraient attribuer à la totalité des épithéliomas médullaires les caractères propres aux cancers à stroma amyloïde. Aussi nous a-t-il paru intéressant de reprendre l'analyse de l'ensemble des observations colligées à l'Institut Gustave-Roussy entre 1933 et 1968, réunissant 34 cas de cancers médullaires répartis comme suit: 24 à stroma amyloïde et 10 sans stroma amyloïde.

Dans le premier groupe, 3 dossiers ont été éliminés parce que trop incomplets. Sur les 21 cas restants, 12 ont été analysés de façon rétrospective, 9 seulement ont fait l'objet d'une étude méthodique. Dans le deuxième groupe, un dossier a également dû être rejeté, et, sur les 9 observations restantes, 8 ont été analysées de façon rétrospective, et un seul a fait l'objet d'une étude prospective.

Un des buts essentiels de ce travail a été de rechercher s'il existe un ensemble de signes cliniques et biologiques qui puisse permettre, comme le fait l'anatomo-pathologie, de distinguer deux catégories distinctes d'épithéliomas médullaires.

Après un bref rappel anatomo-pathologique concernant les deux variétés histologiques, nous étudierons successivement les épithéliomas à stroma amyloïde, puis les cancers médullaires sans stroma amyloïde. Nous verrons enfin quelle conclusion l'on peut en tirer.

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## ANATOMO-PATHOLOGIE

Seuls les principaux caractères anatomo-pathologiques de ces tumeurs peuvent être rappelés ici [1, 25].

*Les épithéliomas médullaires à stroma amyloïde.* Ceux-ci forment dans la glande un nodule en général unique, grossièrement limité, et ne dépassant que tardivement les limites de la capsule thyroïdienne. Au microscope, il s'agit d'une prolifération homogène de petites cellules arrondies ou fusiformes, cytologiquement peu différenciées. Elles sont disposées en îlots, travées ou bandes, sans différenciation vésiculaire ni papillaire. Le stroma est abondant, fibreux, et est semé de nappes hyalines se colorant électivement par les réactifs de l'amylose, les deux réactions les plus utilisées ici étant le rouge Congo alcalin et la fluorescence après thioflavine T. On voit souvent, au contact de ces nappes, une réaction macrophagique et de petites calcifications non lamellaires et à contour polycyclique. Comme le signale Woolner [9], il est des cas où les dépôts amyloïdes manquent dans la tumeur primitive, alors qu'ils sont présents dans les métastases ganglionnaires et vice-versa.

*Le second groupe de tumeurs.* Dans cette groupe les caractères cytologiques et architecturaux sont semblables aux précédents, bien différents de l'envahissement en nappes diffuses et de la cytologie polymorphe des épithéliomas anaplasiques à petites cellules, mais il est impossible d'y démontrer, tant au niveau de la tumeur primitive que des métastases ganglionnaires ou à distance, la présence d'amylose.

Sur le plan histologique, donc, il existe une différence nette selon que le stroma contient ou non des plages amyloïdes, bien que l'architecture et la cytologie des tumeurs soient semblables. Cette amylose doit, bien entendu, être recherchée sur de nombreux prélèvements et par diverses techniques de coloration, les meilleures paraissant le rouge Congo alcalin selon Puchter [26] avec recherche du dichroïsme en lumière polarisée, et la thioflavine T selon Vassar [27] en fluorescence. Le diagnostic microscopique comporte ainsi 2 temps: dans le premier, l'examen élimine un épithélioma différencié ou anaplasique et précise le caractère "médullaire" de la tumeur; dans le second, les techniques spéciales démontrent la présence ou l'absence d'amyloïde dans le stroma. Une distinction anatomo-pathologique nous semble devoir être faite entre ces 2 types d'épithélioma médullaire, les uns banaux, les autres à stroma amyloïde (Fig. 1 et 2).

## LES EPITHELIOMAS MEDULLAIRES A STROMA AMYLOIDE

*La fréquence.* Leur fréquence paraît faible: 24 cas sur un total de 570 cancers du corps thyroïde, examinés à l'Institut Gustave-Roussy de 1933 à 1967, soit 4%. De même, Hazard *et al.* [1] en notent 21 cas sur 600 tumeurs malignes, soit moins de 4%.

*Le sexe.* Dans notre série de 21 cas, 9 concernent des hommes et 12 des femmes. Cette prépondérance féminine est observée par tous les auteurs. Elle est banale dans les cancers de la thyroïde.

*L'âge.* Très variable, allant de 13 à 68 ans; l'âge moyen est de 51 ans. Ces chiffres sont voisins de ceux des grandes statistiques publiées [7, 9, 10].

*Les signes cliniques.* On distingue: d'une part les symptômes propres au cancer: la tumeur quasi constante, les adénopathies cervicales très fréquentes, enfin, les métastases à distance, ganglionnaires surtout, mais aussi viscérales, frappant électivement le squelette, les poumons et le foie.

d'autre part, les symptômes d'emprunt relevant d'associations morbides éventuelles.

*La tumeur.* Celle-ci est de croissance très lente. Il s'agit, dans 15 cas sur 21, d'un nodule thyroïdien *unique*; parfois, cependant, c'est une énorme masse ligneuse et fixée, ainsi que nous l'avons constaté chez un malade dont l'affection évoluait depuis une vingtaine d'années. Fait plus particulier, *la tumeur, quoique indolore spontanément, peut présenter une vive sensibilité lors de la palpation.* Ce signe, recherché chez 10 malades, a été retrouvé 4 fois et nous désirons insister sur la valeur de la constatation d'un tel caractère, qui ne nous paraît pas avoir été signalé précédemment.

*Les adénopathies cervicales sont fréquentes.* Nous les avons retrouvées dans 17 cas sur 21. Volontiers controlatérales ou surtout bilatérales, souvent plus volumineuses que la tumeur thyroïdienne primitive, elles présentent les mêmes caractères de dureté et parfois aussi de sensibilité.

Parmi les autres localisations ganglionnaires, nous avons observé des adénopathies médiastinales dans 3 cas, axillaires dans 2 cas, et même, dans 1 cas, une atteinte de la chaîne lymphatique lombo-aortique coexistant avec une métastase testiculaire.

*Les métastases viscérales.* Elles sont remarquables. Du fait de l'évolution très insidieuse de la maladie, on peut être d'emblée en présence de métastases multiples, parfois révélatrices. C'est notamment le fait des atteintes osseuses, et, dans une de nos observations, c'est

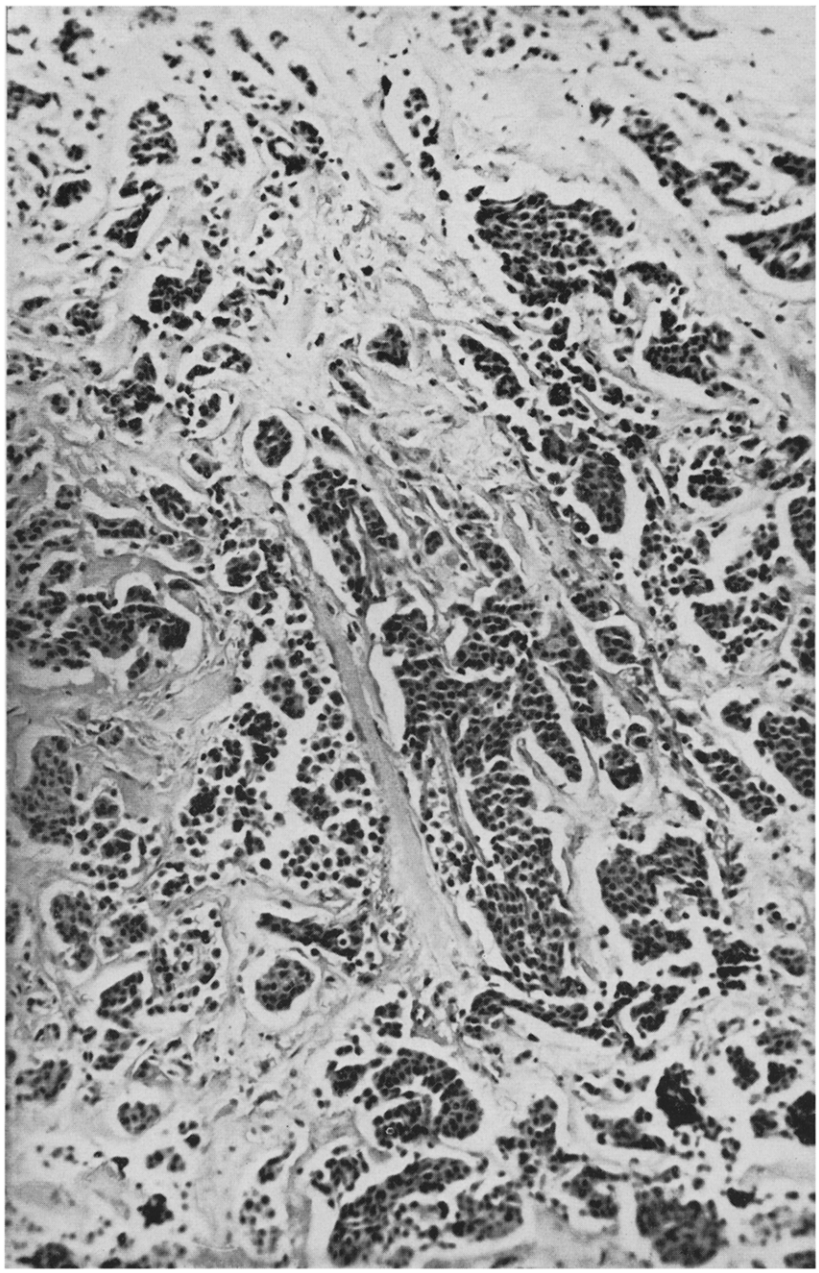

*Fig.* 1. *Epithélioma médullaire à stroma amyloïde.*
*Les plages tumorales, d'architecture solide, sont séparées par des nappes de substance anhiste donnant les réactions de l'amyloïde aux diverses colorations électives. H.P.S.* × 65.

(to face p. 40)

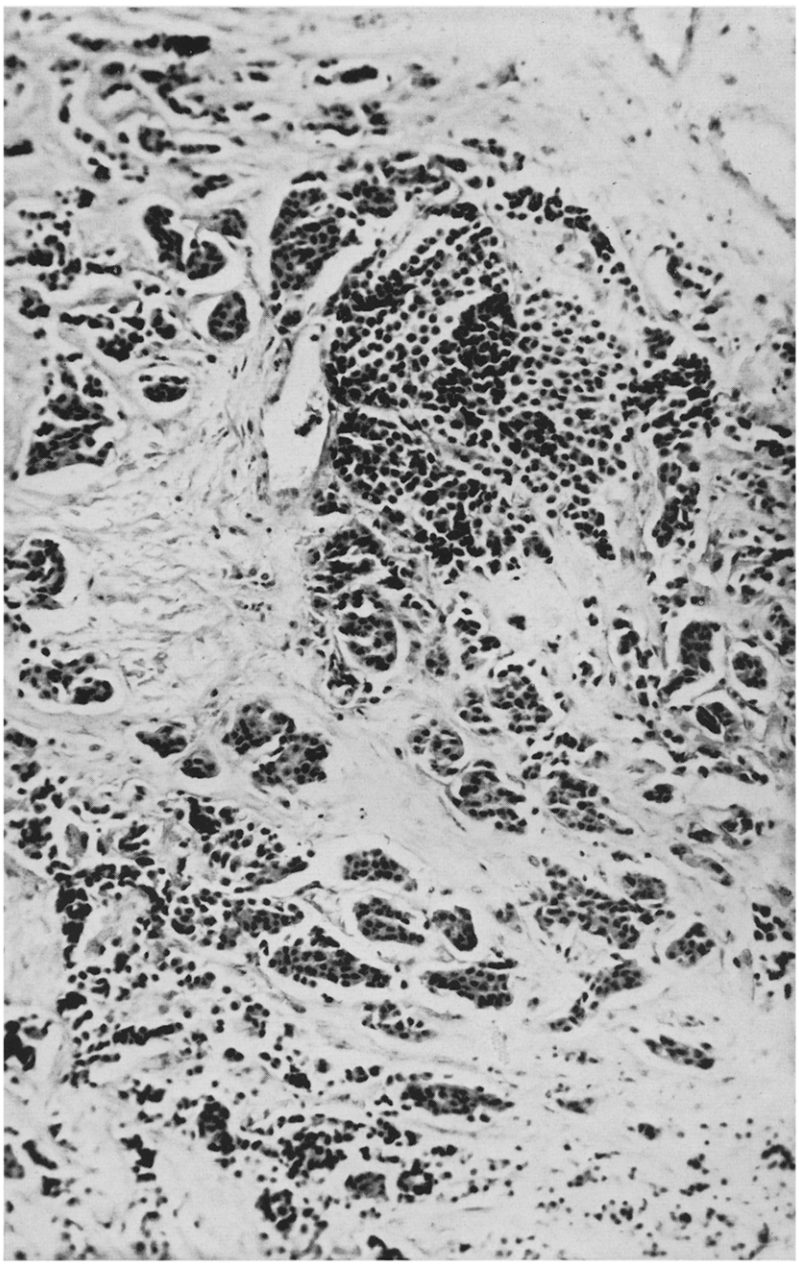

*Fig. 2. Epithélioma médullaire simple.*
*Les caractéristiques cytologiques et architecturales sont semblables, mais le stroma n'est fait que de collagène. La recherche de l'amylose est négative dans toute la tumeur comme dans ses métastases. H.P.S.* × 65.

la localisation secondaire testiculaire qui a permis de découvrir le cancer thyroïdien responsable.

Dans notre série, un seul malade a présenté des métastases pulmonaires, et un autre des métastases cutanées. Par contre, nous avons relevé 3 cas de métastases hépatiques (dont 2 vérifiées histologiquement), fait exceptionnel dans les cancers de la thyroïde, mais qui a déjà été signalé dans les cancers médullaires à stroma amyloïde [7, 28].

*Associations morbides*

Nous n'avons jamais observé de symptôme clinique ni de signe biologique en faveur d'un syndrome carcinoïde, d'un phéochromocytome ou d'une tumeur parathyroïdienne associée. Par contre, chez certains de nos malades, une diarrhée, des névromes muqueux multiples, ou des syndromes malformatifs, isolés ou associés à des degrés divers, ont permis d'évoquer le diagnostic d'épithélioma médullaire à stroma amyloïde du corps thyroïde, dès l'interrogatoire et la simple inspection.

1. *La diarrhée.* Son existence est classique [8–11, 14, 29, 30]. Elle était présente, dès le premier examen, dans 5 observations. Elle n'est apparue, chez les 3 autres malades, que 3, 4 et 13 ans après l'intervention, alors que se développaient des métastases. Il s'agissait, dans tous ces cas, d'une diarrhée purement "motrice", non métabolique, riche en cellulose et en chlorure de sodium. Cette diarrhée s'accompagne d'une importante accélération du transit et évolue parallèlement à la maladie [29]. Elle a disparu après ablation complète de la tumeur et est réapparue dans 3 de nos cas, en même temps que se développaient des récidives ou des métastases.

2. *Les névromes muqueux multiples; les anomalies morphologiques et fonctionnelles qu'ils engendrent; les symptômes malformatifs associés.* Certains auteurs, tels Williams et Pollock [17], ou Gorlin *et al.* [13], ont insisté sur la dysmorphie particulière du visage liée à la présence de névromes muqueux multiples, notamment au niveau des lèvres qui sont massivement augmentées, affectant le type 'négroïde', de la langue, des gencives, des paupières, de la conjonctive, etc. Parfois s'y associe une teinte cyanique du faciès, très évocatrice. Parfois aussi divers troubles intestinaux que, dans une de nos observations, l'histologie a montré être dus à "une hyperplasie nerveuse et ganglionnaire considérable intéressant les plexus de Meissner et d'Auerbach". Ces faits se rapprochent de ceux décrits par certains auteurs sous le terme de "ganglio-neuromatose intestinale diffuse" [15, 16].

D'autres malformations peuvent également être associées. Une de nos observations (dossier 12.68.5586 AG) concernait une jeune fille de 15 ans, porteuse d'un épithélioma médullaire à stroma amyloïde du corps thyroïde, avec adénopathies cervicales controlatérales; son faciès était caractéristique, cyanique, avec des lèvres de type 'négroïde' et une langue épaisse. On notait en outre une amyotrophie considérable et diffuse, une pied creux bilatéral, un infantilisme, et de fréquentes crises de diarrhée. Ce tableau clinique a lui seul permettait d'affirmer le diagnostic [31].

Le caractère fréquemment familial de ces tumeurs a été signalé; à cet égard, nous rapporterons une observation dans laquelle un épithélioma médullaire à stroma amyloïde a été observé chez la grand-mère, la mère et la fille. La mère, L.S. (dossier 7.31.946) présentait, à l'âge de 24 ans, une tuméfaction cervicale gauche se développant progressivement. L'année suivante, en 1946, une lobectomie est pratiquée, suivie de l'administration de 100 millicuries d'iode radioactif. La malade, suivie régulièrement depuis, est actuellement en bonne santé. En septembre 1965, on est amené à examiner sa fille, L. Cl. (dossier 7.65.748), alors âgée de 19 ans, qui présente deux petits nodules du lobe gauche du corps thyroïde, connus depuis au moins 6 mois, ne grossissant pas et s'avérant hypofixants à la scintigraphie. Une hémithyroïdectomie, avec curage ganglionnaire, est pratiquée, suivie de cobalthérapie (6.000 rads). La patiente, revue périodiquement à la consultation, est actuellement en excellente santé. L'interrogatoire nous apprenait alors que la grand-mère, M.I., avait été opérée d'une tumeur cervicale à l'âge de 52 ans, en juin 1944, à la Fondation Curie. Les coupes communiquées, confrontées avec celles de la fille et de la petite fille, montraient un épithélioma médullaire à stroma amyloïde du corps thyroïde, identique aux deux tumeurs précédentes. Ces formes familiales ont déjà été décrites, mais, à notre connaissance, c'est la première dans laquelle on peut trouver le cancer dans 3 générations successives. Al Saadi et Lieberman [20] ont récemment souligné l'intérêt de l'analyse des chromosomes des lymphocytes circulants dans le cancer médullaire qui aurait révélé un pourcentage élevé d'aneuploïdie, non seulement chez les malades, mais encore chez différents membres de la famille, notamment les collatéraux et les descendants. Cependant, chez cette famille,

l'étude des chromosomes des lymphocytes circulants de la mère (L.S.) n'a montré aucune anomalie du caryotype.*

*La présence de thyrocalcitonine.* La présence de TCT dans le sang et dans la tumeur a récemment été signalée [21], et cette observation a depuis été confirmée [22–24]. Nous l'avons recherchée dans 7 cas [22]. Nous avons retrouvé dans le tissu tumoral (thyroïde ou ganglion cervical) de tous les malades porteurs d'un épithélioma médullaire à stroma amyloïde, une très forte activité hypocalcémiante et hypophosphatémiante (de concentration 100 à 600 fois supérieure à celle du tissu thyroïdien normal par unité de poids). Chez 4 patients sur 7, nous avons également observé une forte activité hypocalcémiante du sérum, reflet de la sécrétion de thyrocalcitonine par les tumeurs médullaires à stroma amyloïde. L'un de ces cas paraît très démonstratif: la TCT avait disparu après l'intervention, et sa réapparition dans le sérum coïncide avec le développement de métastases. Dans les 3 autres cas où la TCT était absente du sérum, l'exérèse avait été réalisée de façon complète, et il n'existait aucun signe d'évolution. Parmi ces 3 malades, nous avons vu apparaître, dans 1 cas, un taux plasmatique élevé de TCT, parallèlement à une récidive ganglionnaire.

## LES EPITHELIOMAS MEDULLAIRES SANS STROMA AMYLOIDE

*La fréquence.* Leur fréquence est très faible. On n'en compte que 10 cas pour un total de 570 cancers thyroïdiens colligés à l'Institut Gustave-Roussy entre 1933 et 1967, soit un pourcentage d'environ 2%.

*Le sexe.* L'Homme paraît être plus souvent atteint, dans notre série tout au moins, puisque, parmi les 9 cas retenus, l'on dénombre 6 hommes pour 3 femmes.

*L'âge.* Il varie de 33 à 69 ans. L'âge moyen est de 50 ans: il est donc pratiquement superposable à celui observé dans les épithéliomas médullaires à stroma amyloïde.

Aucune notion géographique ni familiale n'a jamais pu être incriminée dans cette variété de tumeurs.

*Les signes cliniques.* Ils sont ceux liés au cancer ou à sa dissémination, à l'exclusion de toute manifestation ou association morbide.

*La tumeur.* Dans 8 cas sur 9, l'on retrouvait un nodule thyroïdien unique, de taille variable, sans caractère particulier. Dans un cas seulement, il s'agissait d'une volumineuse tumeur fixée, dont on ne pouvait déterminer la nature exacte, thyroïdienne ou ganglionnaire. Mais, contrairement aux épithéliomas médullaires à stroma amyloïde, ces tumeurs ne sont pas sensibles à la palpation et ont une croissance rapide. En effet, toutes, sauf une, avaient été remarquées depuis moins d'un an et avaient augmenté de volume assez rapidement.

*Les adénopathies.* Des adénopathies cervicales, jugulaires ou sus-claviculaires ont été notées dans 5 cas sur 9. Aucune autre localisation ganglionnaire n'a été signalée.

*Les métastases.* Elles semblent assez fréquentes et leur localisation est celle habituellement notée dans les cancers thyroïdiens. Nous avons observé, 2 fois, des atteintes cérébrales secondaires. Dans un cas, l'autopsie a révélé l'existence de métastases hépatiques.

*Association morbide.* Aucune n'a été rencontrée.

*La biologie.* Au point de vue biologique, nous n'avons pas retrouvé de thyrocalcitonine dans le sérum d'une malade porteuse d'un cancer médullaire sans stroma amyloïde, alors qu'elle présentait une volumineuse récidive tumorale cervicale et plusieurs métastases osseuses [22]. Il semble donc que, contrairement aux épithéliomas médullaires à stroma amyloïde, les cancers médullaires sans stroma amyloïde ne sécrètent pas de TCT. Cependant, cette conclusion n'est basée que sur 1 cas.

Ainsi, des différences apparaissent-elles manifestes entre ces deux types de tumeurs. Les cancers médullaires sans stroma amyloïde se distinguent notamment par la prédominance, semble-t-il, de leur survenue dans le sexe masculin, l'absence d'antécédents familiaux et, au point de vue clinique, par l'absence de sensibilité de la tumeur ou des ganglions lors de la palpation, ainsi que par l'absence d'association morbide. En pratique, les cancers médullaires sans stroma amyloïde sont cliniquement plus proches des épithéliomas de type anaplasique. L'absence de thyrocalcitonine pourrait constituer un argument biologique différentiel supplémentaire entre ces deux types de cancers médullaires.

L'ensemble de ces différences cliniques et biologiques a d'autant plus d'importance pour différencier ces deux types de cancer que, sur le plan histopathologique, des erreurs sont possibles. La présence de stroma amyloïde peut parfois s'observer uniquement soit dans la tumeur primitive, soit dans des métastases [9]. L'une de nos observations illustre ce fait.

Monsieur A. (dossier 7.67.6515) présentait un cancer médullaire à stroma amyloïde de la thyroïde avec métastase testiculaire et métas-

*Examen pratiqué par le Professeur J. Lejeune et le Docteur R. Berger (Hôpital des Enfants-Malades, Paris).

*Tableau* 1. *Taux de survie des épithéliomas médullaires avec et sans stroma amyloïde*

| | | Survie à 3 ans | à 5 ans | à 10 ans |
|---|---|---|---|---|
| Epithéliomas médullaires à stroma amyloïde | Femmes | 6/7 | 4/5 | 2/5 |
| | Hommes | 8/8 | 3/4 | 2/3 |
| | Total | 14/15 | 7/9 (77%) | 4/8 (50%) |
| Epithéliomas médullaires sans stroma amyloïde | Femmes | 3/3 | 2/3 | 1/2 |
| | Hommes | 1/6 | 0/6 | 0/5 |
| | Total | 4/9 | 2/9 | 1/7 |

tase dans les ganglions lombo-aortiques. Dix-huit mois après traitement, un nodule sous-cutané apparaît dans la région abdominale. Sa biopsie montre qu'il s'agit d'une métastase, mais on ne peut pas mettre en évidence de stroma amyloïde. Ceci montre que le diagnostic histologique est difficile et qu'il est prudent d'étudier plusieurs prélèvements avant de conclure. En particulier, l'absence d'amylose sur un prélèvement extrathyroïdien ne devrait pas suffire à exclure l'hypothèse d'un cancer médullaire à stroma amyloïde.

*Evolution*

L'ensemble de ces caractères différentiels a un intérêt clinique car, sur le plan évolutif, la différence entre ces deux types de cancer paraît nette, comme le montre le Tableau 1 qui compare la survie à 3, 5 et 10 ans en fonction du type histologique de la tumeur.

1. Dans le cas des épithéliomas médullaires à stroma amyloïde: après 3 ans, sur 15 malades, 14 sont encore en vie. Parmi les 9 patients ayant plus de 5 ans de recul, 7 sont toujours vivants, et 4 des 8 cas ayant plus de 10 ans de recul sont actuellement en bonne santé.

2. Dans le cas des épithéliomas médullaires sans stroma amyloïde: après 3 ans, sur 9 malades, 4 sont encore en vie; après 5 ans, 2 sont toujours vivants, et, parmi les 7 cas ayant plus de 10 ans de recul, il ne reste qu'une seule survivante, d'ailleurs porteuses de métastases multiples.

3. L'analyse statistique de ces résultats montre que les différences de survie à 3 ans, 5 ans et même 10 ans sont, en dépit du faible nombre de cas, significatives au seuil de 5%. Les différences de survie entre hommes et femmes ne sont pas significatives. La méthode du $\chi^2$ n'étant pas applicable dans ce cas, nous avons eu recours au calcul direct des probabilités [32].

La comparaison avec les autres types de

*Tableau* 2. *Taux de survie selon la variété anatomo-pathologique*

| Type anatomopathologique | Nombre de cas +5 ans | Décédés | Perdus de vue | Taux de survie à 5 ans | Nombre de cas à 10 ans | Décédés | Perdus de vue | Taux de survie à 10 ans |
|---|---|---|---|---|---|---|---|---|
| Vésiculaires + Papillaires | 116 | 13 | 13 | (87%) 103 | 60 | 15 | 10 | (70%) 50 |
| Adénomes malins | 22 | 3 | 3 | (84%) 19 | 10 | 2 | 1 | (77%) 9 |
| Trabéculaires | 27 | 6 | 0 | (77%) 27 | 14 | 4 | 4 | (60%) 10 |
| Epidermoïdes et Métaplasiques | 6 | 3 | 0 | (3/6) | 3 | 3 | 0 | 0 |
| Anaplasiques | 53 | 48 | 3 | (4%) 50 | 34 | 32 | 1 | (3%) 33 |

Les taux de survie ont été calculés après avoir éliminé les malades perdus de vue.

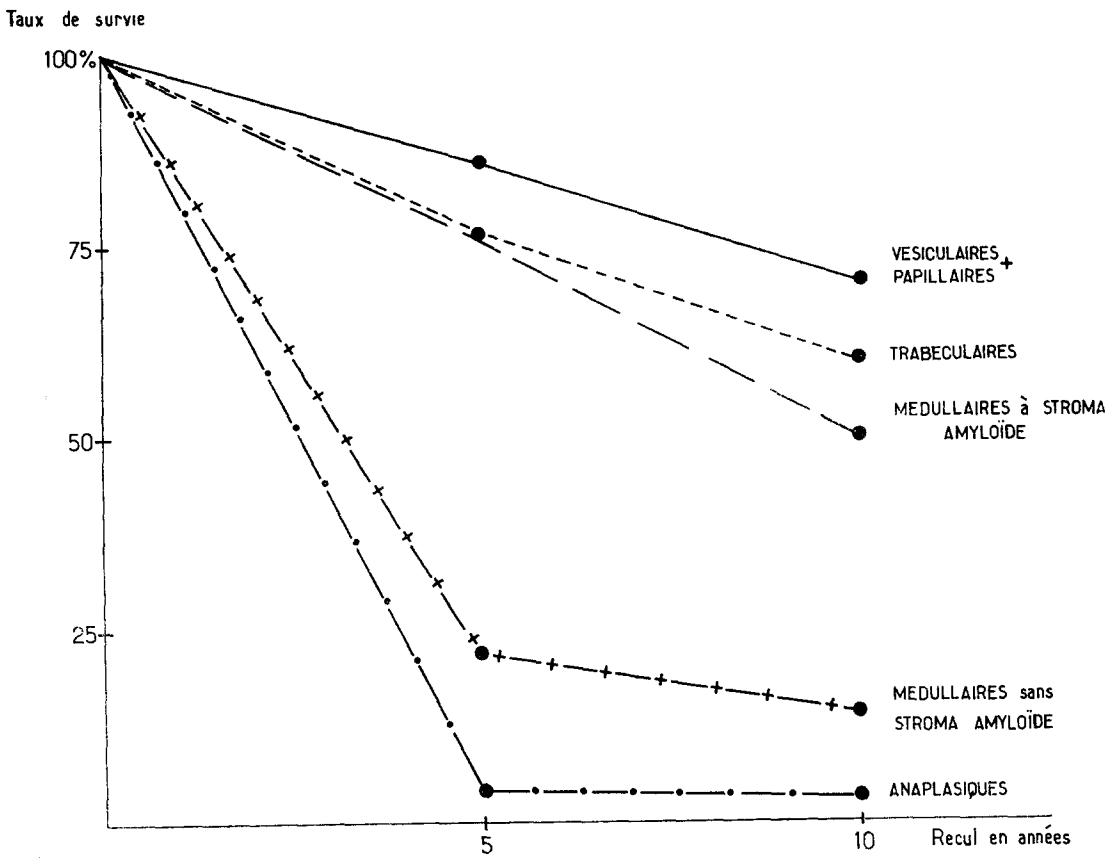


*Fig. 3. Taux de survie à 5 ans et à 10 ans des différents types histopathologiques de cancers du corps thyroïde.*

cancer thyroïdien (Tableau 2 et Fig. 3) montre qu'au point de vue évolutif, les épithéliomas médullaires à stroma amyloïde sont très proches des épithéliomas trabéculaires, et que les cancers médullaires sans stroma amyloïde s'inscrivent entre les trabéculaires et les anaplasiques de notre série [33].

## CONCLUSION

Si les épithéliomas médullaires à stroma amyloïde représentent une entité à la fois clinique, biologique et anatomo-pathologique, il semble que les signes des cancers médullaires sans stroma amyloïde ne leur permettent pas d'occuper une place particulière dans la nosologie. En effet, leur pronostic est peu différent de celui des cancers indifférenciés et leur épidémiologie, ainsi que leur symptomatologie clinique et biologique, ne présentent aucun caractère particulier.

**Remerciements** — Nous tenons à remercier le Docteur J. Roujeau qui a assuré la surveillance de la plupart des malades inclus dans ce travail.

Nous sommes reconnaissants au Docteur O. Schweisguth qui a accepté de relire notre texte et nous a fait profiter de ses critiques.

Nous remercions le Professeur G. Milhaud et le Docteur G. Coutris qui ont effectué les dosages de thyrocalcitonine chez nos malades, et Monsieur J. Chavaudra qui nous a donné de précieux conseils pour certains analyses statistiques pratiquées sur nos résultats.

## RESUME

*A côté du cancer médullaire à stroma amyloïde du corps thyroïde, entité maintenant bien connue, existent des aspects histo-pathologiques de cancer médullaire sans stroma amyloïde, relativement peu étudiés. Sur le plan histo-pathologique, des confusions sont possibles car le stroma amyloïde peut être présent dans la tumeur primitive, et non dans les métastases, et vice-versa.*

*Afin de rechercher si ces deux aspects histo-pathologiques correspondent à une ou deux entités morbides, les caractères cliniques et biologiques de 24 cancers médullaires à stroma amyloïde de la thyroïde et de 10 cancers médullaires sans stroma amyloïde de la thyroïde, examinés à l'Institut Gustave-Roussy entre 1933 et 1968, ont été comparés.*

*L'étude des épithéliomas médullaires à stroma amyloïde a montré que la masse tumorale ainsi que les adénopathies sont parfois sensibles à la palpation, et que l'extension gan-*

*glionnaire est souvent très diffuse. Elle a confirmé le caractère familial de l'affection (nous avons notamment observé le cas d'une famille dans laquelle ce type de cancer apparaît dans trois générations), ainsi que l'existence d'associations maintenant classiques, notamment avec une diarrhée évoluant parallèlement à la maladie, avec des névromes multiples des muqueuses, et avec des malformations à type d'amyotrophie.*

*Les caractères cliniques des cancers médullaires sans stroma amyloïde sont différents. Nous avons noté dans notre série la prédominance chez l'homme. La tumeur et les adénopathies ne sont pas douloureuses à la palpation. Aucune association morbide n'a été relevée. L'extension ganglionnaire et métastatique est semblable à celle des autres cancers thyroïdiens.*

*Sur le plan biologique, nous avons observé la présence de thyrocalcitonine dans la tumeur ou le plasma de tous les cancers médullaires à stroma amyloïde dans lesquels nous l'avons recherchée. Dans un cas de cancer médullaire sans stroma amyloïde, nous n'avons pas mis en évidence de thyrocalcitonine dans le plasma.*

*La survie des cancers à stroma amyloïde est voisine, dans notre série, de celle des épithéliomas trabéculaires de la thyroïde. La survie des cancers médullaires sans stroma amyloïde est beaucoup plus courte, proche de celle des cancers anaplasiques de la thyroïde.*

*Les cancers médullaires sans stroma amyloïde diffèrent donc de ceux avec stroma amyloïde au point de vue clinique, biologique et pronostique, et il est impossible de réunir ces deux variétés histo-pathologiques dans une même entité clinique.*

## SUMMARY

*Besides the medullary cancer with an amyloid stroma of the thyroid which is a well known entity there are other histopathological aspects of medullary cancer without amyloid stroma which have been relatively little studied. There can be confusion from histo-pathological view-point because the amyloid stroma can be present in the primitive tumour and absent in metastases or vice versa.*

*To find out whether these two pathological aspects correspond to one or two morbid entities, the clinical and biological aspects of* 24 *medullary cancers with amyloid and* 10 *without amyloid which were observed at the Gustave Roussy Institute between* 1933 *and* 1968 *have been compared.*

*The study of the medullary carcinomas with amyloid stroma shows that the tumour mass as well as the lymph nodes are tender and that the diffuse adenopathy is often very difficult to delineate. It confirms the familial character of the disease (we have the case of a family in which this kind of cancer appears in three generations), as well as the existence of classical associations, especially a diarrhoea which fluctuates with the disease, multiple neuromas of the mucous membranes and malformations of the amyothropic type.*

*The clinical characters of medullary cancers without amyloid stroma are different. They predominate in males. Tumours and lymph nodes are not tender. No associated signs have been observed. The lymph nodes and distant metastases are similar to those of other thyroid cancers.*

*From a biological view-point we observed the presence of thyrocalcitonine in the tumour or the plasma of all patients with medullary cancers with amyloid stroma in which it was looked for. In a case of medullary cancer without amyloid stroma there was no thyro-calcitonine in the plasma.*

*The survival time in cancers with amyloid stroma is similar of our series with that of other epitheliomas of the thyroid. The survival in medullary cancers without amyloid stroma is much shorter, approaching that of the anaplastic cancers of the thyroid.*

*Consequently medullary carcinomas with and without amyloid stroma must be considered as separate entities since they differ clinically, biologically and in regard to prognosis.*

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# Chromosome Studies on Workers Exposed to Atmospheric Benzene

## The Possible Influence of Age

I. M. TOUGH, P. G. SMITH, W. M. COURT BROWN and D. G. HARNDEN*

*Medical Research Council, Clinical and Population Cytogenetics Research Unit, Western General Hospital, Edinburgh, U.K.*

### INTRODUCTION

In a preliminary report published in 1965 Tough and Court Brown [1] reported that individuals who have been exposed to benzene in the atmosphere, but who have no haematological disorder, may show structural chromosome aberrations in lymphocytes cultured from the peripheral blood. The data in this previous report demonstrated a small but significant increase in the percentage of cells with unstable chromosome aberrations ($C_u$ cells) in a group of men exposed to benzene as compared with a small on-site control group of men taken together with a number of adult males randomly selected from the general population.

Pollini and his co-workers in 1964 published a series of papers [2–4] on chromosome damage in man and its relationship to blood disorders caused by exposure to benzene.

Forni [5] has presented data on cytogenetic studies on patients who have recovered from benzene-induced blood disorders and on subjects who have been exposed to benzene but who did not show signs of toxicity, and demonstrates a slight increase in chromosome aberrations in both groups.

Since the time of the preliminary report by Tough and Court Brown we have studied workers from two other factories where there has been exposure to benzene in the atmosphere and the data from all three groups are considered in this report.

### SUBJECTS STUDIED

Groups of individuals have been examined from three different factories in which benzene had been present in the atmosphere. In factories 1 and 2 (Groups 1 and 2 workers) similar products were manufactured and benzene had been used as a solvent in such a way that large surface areas of liquid were open to the atmosphere. In Factory 3 (Group 3 workers), however, exposure had been intermittent and probably small in amount, and was due to the leakage of benzene from a closed distillation plant. In none of the factories is the exact exposure known nor are there any accurate histories on the relative exposures of the different workers. None of the men included in the series are known to have received therapeutic levels of X-irradiation.

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*Present address: Department of Cancer Research, University of Birmingham, Birmingham, U.K.

*Table* 1a (*exposed*).

| Group 1 | | | | | | | Group 2 | | | | | | | Group 3 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age at study (years) | Years exp. to benzene | Years since exp. to benzene | $C_s$ | $C_u$ | Total R+D | Cells with R or D | Age at study (years) | Years exp. to benzene | Years since exp. to benzene | $C_s$ | $C_u$ | Total R+D | Cells with R or D | Age at study (years) | Years employed at factory* | $C_s$ | $C_u$ | Total R+D | Cells with R or D |
| 23 | 3 | 3 | 0 | 0 | 0 | | 43 | 7 | 6 | 2 | 0 | 0 | | 22 | 7 | 0 | 0 | 0 | |
| 26 | 2 | 2 | 2 | 0 | 0 | | 45 | 17 | 4 | 0 | 0 | 0 | | 23 | 2 | 0 | 0 | 0 | |
| 28 | 5 | 2 | 1 | 0 | 0 | | 46 | 13 | 4 | 1 | 1 | 1 | 1 | 27 | 3 | 0 | 0 | 0 | |
| 40 | 15 | 3 | 1 | 0 | 0 | | 49 | 7 | 4 | 1 | 5 | 1 | 1 | 37 | 14 | 0 | 0 | 0 | |
| 41 | 11 | 2 | 1 | 1 | 0 | | 54 | 11 | 4 | 0 | 1 | 1 | 1 | 39 | 15 | 0 | 0 | 0 | |
| 43 | 12 | 3 | 0 | 2 | 1 | 1 | 54 | 4 | 4 | 0 | 0 | 0 | | 41 | 12 | 1 | 0 | 0 | |
| 44 | 1 | 2 | 0 | 2 | 0 | | 58 | 23 | 4 | 1 | 2 | 0 | | 42 | 11 | 0 | 1 | 1 | 1 |
| 45 | 8 | 2 | 1 | 0 | 0 | | 60 | 17 | 4 | 0 | 1 | 0 | | 42 | 15 | 0 | 0 | 0 | |
| 45 | 16 | 3 | 3 | 1 | 1 | 1 | 60 | 21 | 4 | 1 | 0 | 0 | | 47 | 13 | 0 | 0 | 0 | |
| 49 | 7 | 2 | 0 | 0 | 0 | | 60 | 20 | 4 | 1 | 0 | 0 | | 49 | 7 | 1 | 0 | 0 | |
| 50 | 17 | 3 | 1 | 1 | 0 | | 61 | 17 | 4 | 0 | 4 | 3 | 3 | 49 | 2 | 0 | 0 | 0 | |
| 51 | 7 | 2 | 0 | 3 | 3 | 2 | 65 | 20 | 4 | 1 | 5 | 5 | 3 | 49 | 4 | 1 | 1 | 0 | |
| 54 | 2 | 2 | 2 | 4 | 2 | 2 | | | | | | | | 56 | 6 | 0 | 0 | 0 | |
| 54 | 14 | 2 | 0 | 1 | 0 | | | | | | | | | 62 | 26 | 1 | 2 | 1 | 1 |
| 55 | 7 | 2 | 1 | 2 | 0 | | | | | | | | | 63 | 23 | 1 | 0 | 0 | |
| 56 | 7 | 3 | 1 | 1 | 0 | | | | | | | | | 63 | 25 | 0 | 1 | 0 | |
| 59 | 8 | 2 | 1 | 2 | 1 | 1 | | | | | | | | 64 | 20 | 2 | 0 | 0 | |
| 61 | 7 | 2 | 2 | 3 | 0 | | | | | | | | | 64 | 17 | 1 | 1 | 0 | |
| 61 | 6 | 2 | 1 | 4 | 2 | 2 | | | | | | | | 65 | 22 | 2 | 0 | 0 | |
| 64 | 9 | 2 | 1 | 3 | 0 | | | | | | | | | 69 | 18 | 0 | 0 | 0 | |

*Table* 1b (*controls*).

| Group 1 | | | | | | | Group 2 | | | | | | | Group 3 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age at study (years) | Years exp to. benzene | Years since exp. to benzene | $C_s$ | $C_u$ | Total R+D | Cells with R or D | Age at study (years) | Years exp. to benzene | Years since exp. to benzene | $C_s$ | $C_u$ | Total R+D | Cells with R or D | Age at study (years) | Years employed at factory* | $C_s$ | $C_u$ | Total R+D | Cells with R or D |
| 20 | | | 0 | 0 | 0 | 0 | 42 | | | 0 | 1 | 1 | 1 | 30 | | 2 | 2 | 1 | 1 |
| 33 | | | 0 | 2 | 1 | 1 | 45 | | | 0 | 0 | 0 | 0 | 36 | | 0 | 0 | 0 | 0 |
| 34 | | | 0 | 0 | 0 | 0 | 51 | | | 1 | 0 | 0 | 0 | 39 | | 0 | 0 | 0 | 0 |
| 34 | | | 0 | 0 | 0 | 0 | 52 | | | 1 | 1 | 1 | 1 | 39 | | 0 | 0 | 0 | 0 |
| 53 | | | 2 | 1 | 0 | 0 | 52 | | | 0 | 3 | 3 | 2 | 44 | | 0 | 0 | 0 | 0 |
| | | | | | | | 59 | | | 1 | 5 | 2 | 2 | | | | | | |

$C_s$ Cells with stable chromosome rearrangements.
$C_u$ Cells with unstable chromosome rearrangements.
R Ring chromosome
D Dicentric chromosome
* In Group 3 the years worked in the factory is equivalent to the time of any exposure to benzene.

*Group* 1

The details of this group have already been published [1]. It consisted of 20 men who had been exposed to benzene until 1962, for periods varying from 1–20 years, and also 5 men who had been employed for similar lengths of time, working in an area of the factory where benzene had not been in use. Samples were obtained from 14 of these men in November 1964, and from the remaining 11 in January 1965.

*Group* 2

A total of 18 men were examined from a factory in which benzene was used as a solvent until April 1962. Blood samples were taken in January 1966. Twelve of these individuals had worked for periods varying from 6 to 25 years in an area of the factory where benzene was known to have been present. The other 6 individuals, of comparable age, had worked in the factory for similar lengths of time (16–24 years), but in an area where benzene had not been in use.

*Group* 3

In July 1965, samples were obtained from 20 men who were working in a closed distillation plant where benzene was known to have been present in the atmosphere at intervals up to the time of study. It is possible that the exposure in this factory was less than in 1 and 2. The men had worked in the plant for periods varying from 2 to 26 years. Five further individuals who worked in the same factory, but were never employed in the distillation plant, were examined for control purposes.

## METHODS

The lymphocytes were cultured by a modification of the method of Moorhead *et al.* [6] and in all instances the cultures were harvested after between 40 and 50 hr inclusive of 3 hr with diacetylmethylcolchicine (colcemid: Ciba). Buckton and Pike [7] have established that standardization of culture time is important in the scoring of abnormal cells in lymphocyte cultures.

One hundred cells were counted and analysed from each individual, with all the observations made by the same person. At the time of scoring the cytologist did not know which individuals had been exposed to benzene. The number of cells containing unstable aberrations ($C_u$ cells) and those with stable aberrations ($C_s$ cells) were scored after the method of Buckton and Pike [7].

*Results*

Observations on the exposed workers and on-site controls are shown individually in Table 1. Dispersion tests for homogeneity have been performed within each exposed and control group, assuming a Binomial distribution of $C_u$ and $C_s$ cells and a Poisson distribution of ring and dicentric chromosomes. Groups 1 and 3 would seem to be homogeneous with regard to $C_u$ cells, $C_s$ cells and rings and dicentrics but there is evidence of heterogeneity in the exposed workers in Group 2 for both $C_u$ cells ($\chi^2$ (11 d.f)=27·5; $P<0·01$) and rings and dicentrics ($\chi^2$ (11 d.f)=29·4; $P<0·01$) and amongst the Group 2 controls with respect to $C_u$ cells ($\chi^2$ (5 d.f)=11·8; $P<0·05$). The findings are summarized in Table 2 along with the findings on a group of males of comparable

*Table* 2.

| | | Total cells | $C_u$ cells (No.) | $C_u$ cells (%) | $C_s$ cells (No.) | $C_s$ cells (%) | Rings and dicentrics (No.) | Rings and dicentrics per 1000 cells |
|---|---|---|---|---|---|---|---|---|
| Group 1 | Exposed | 2000 | 30 | 1·50 | 19 | 0·95 | 10 | 5·0 |
| | Controls | 500 | 3 | 0·60 | 2 | 0·40 | 1 | 2·0 |
| Group 2 | Exposed | 1200 | 19 | 1·58 | 8 | 0·67 | 11 | 9·2 |
| | Controls | 600 | 10 | 1·67 | 3 | 0·50 | 7 | 11·7 |
| Group 3 | Exposed | 2000 | 6 | 0·30 | 10 | 0·50 | 2 | 1·0 |
| | Controls | 500 | 2 | 0·40 | 2 | 0·40 | 1 | 2·0 |
| General population sample [8] | | 1060 | 6 | 0·57 | 9 | 0·85 | 0 | 0·0 |

age selected at random from the general population [8]. In this table the percentages of cells containing stable abnormalities ($C_s$) or unstable abnormalities ($C_u$) are recorded but since the former will only be recognized if the products of a rearrangement differ from the normal karyotype, it must be borne in mind that the $C_u$ cells provide a more reliable indication of the action of a chromosome damaging agent. The number of ring and dicentric chromosomes observed is also recorded since it is known that such chromosomes are sensitive indicators of exposure to radiation *in vivo* and may therefore be of special interest when exposure to other chromosome damaging agents is being considered.

The significant increase in the percentage of $C_u$ cells in the exposed workers of group 1 has already been reported [1]. It is clear that such a difference is not present in groups 2 and 3. In group 2, however, the frequency of aberrations observed in the exposed workers is higher than one would expect by comparison with the general population but it does not differ from the level in the on-site controls which is also unusually high. In group 3 the level of aberrations is approximately the same in the exposed workers and on-site controls and neither differ significantly from the general population.

Perhaps the most striking feature of Tables 1 and 2 is the high frequency of rings and dicentrics in Group 1 exposed workers and in both control and exposed workers in Group 2. The level in Group 1 is significantly higher than that observed by Court Brown *et al.* [8] in a sample from the general population of Edinburgh (Table 2) ($\chi^2$ (1 d.f)$=3{\cdot}87$; $P<0{\cdot}05$). The overall levels in Group 2 are also considerably raised although formal significance testing is complicated by the lack of homogeneity in the group.

## DISCUSSION

The interpretation of these results is difficult and while the frequency of chromosome aberrations, particularly of rings and dicentrics, is much higher than has been observed in any normal population, it now appears that the correlation between exposure to benzene and an excess of chromosome aberrations in blood cells is not a simple one. Exposure to benzene is probably one of the factors contributing to these anomalous results but it does appear that it is not the only factor. In Group 3 the results show that workers and on-site controls do not differ from the general population control. This could be due to the different nature of the work in this factory and may represent only a low level exposure of these workers.

In group 2 the principal anomaly is that while the exposed workers appear to have an increased incidence of $C_u$ cells, the on-site controls also have elevated levels. One possible explanation would be that these controls were also exposed to atmospheric benzene and therefore their working history was reinvestigated. One subject (a 59-year old male) was discovered to have used benzene for cleaning purposes roughly once a week for an 8-year period ending 15 yr prior to the date of study. His exposure, though at some time in the past, may therefore have been considerable and it is of interest that 5 $C_u$ cells including two with dicentric chromosomes were found in 100 cells studied. While none of the others had worked in the area where the major benzene exposure occurred they all at some time worked in the same building. Two had worked there for 10 yr but had been transferred to another building 10 years before samples were taken. Two had been there for 9 yr but there had been no possibility of benzene exposure for 10 yr and 4 yr respectively prior to sampling. The fifth subject had worked there for only two years ending 22 yr before the samples were taken. Because of the highly volatile nature of benzene it is concluded that the possibility that these subjects had been exposed to some level of atmospheric benzene at some time in the past does exist. No measurements are available but the physical relationship of the different working areas suggests that any exposure must have been less for the controls and moreover that in four out of six cases any possibility of exposure ceased ten or more years prior to the taking of blood samples.

In the case of Factory 1 measurements are available showing that levels of between 25 ppm and 150 ppm benzene in the atmosphere were recorded in the high exposure area. Conditions in Factory 2 were similar and it is likely that the exposure levels were similar. In Factory 3 the level of atmospheric contamination was approximately 12 ppm of benzene.

Another factor which may contribute to these results emerges from a consideration of the data. The Group 2 controls were matched for age while the Group 1 controls are con-

*Table 3. Chromosome aberations in a random sample of the general population* [8].

| Age group (years) | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total cells | $C_u$ (No.) | (%) | $C_s$ (No.) | (%) | Total cells | $C_u$ (No.) | (%) | $C_s$ (No.) | (%) |
| 15–24 | 540 | 0 | 0·00 | 4 | 0·74 | 510 | 3 | 0·59 | 6 | 1·18 |
| 25–34 | 660 | 4 | 0·61 | 8 | 1·21 | 720 | 4 | 0·56 | 7 | 0·97 |
| 35–44 | 536 | 6 | 1·12 | 5 | 0·93 | 480 | 6 | 1·25 | 6 | 1·25 |
| 45–54 | 510 | 4 | 0·78 | 4 | 0·78 | 540 | 12 | 2·22 | 6 | 1·11 |
| 55–64 | 598 | 3 | 0·50 | 7 | 1·17 | 690 | 11 | 1·60 | 8 | 1·16 |
| 65–74 | 2130 | 29 | 1·36 | 42 | 1·97 | 2039 | 38 | 1·86 | 22 | 1·08 |
| 74+ | 980 | 14 | 1·43 | 26 | 2·65 | 1487 | 31 | 2·08 | 38 | 2·56 |

siderably younger than the Group 1 exposed workers. Casual examination of Table 1 suggests that more $C_u$ cells occur in the higher age groups and it was decided to investigate the effect of age on the incidence of structural chromosome aberrations. The simple product moment correlation co-efficient has been calculated, for each of the exposed groups, relating the number of $C_u$ cells to the age of the individual at the time of study. There is a significant positive correlation in Group 1 ($r=0·705$ d.f.$=18$; $P<0·001$) but the increase with age is not statistically significant in Group 2 [$r$ (10 d.f.)$=0·34$] or Group 3 [$r$ (18 d.f.)$=0·32$]. However, the pooled estimate of correlation for all three groups is significant ($r=0·406$ d.f.$=46$; $P<0·01$). There is no correlation between the number of $C_u$ cells and the years of exposure to benzene in any of the groups. Re-examination of the data given by Court Brown *et al.* [8] for a general population suggests that a correlation with age can again be demonstrated. Table 3 shows the data of Court Brown. The figures for early and late cultures have been combined to provide adequate numbers—there being no marked differences between the results at the two culture times. For the males there is a significant increase in the proportion of $C_u$ cells with increasing age ($\chi^2$ (1 d.f.-trend)$=9·07$; $P<0·01$, $\chi^2$ (5 d.f. departures from linearity) $=3·76$; not significant). A similar effect may be demonstrated for $C_s$ cells and the same significant increases in the number of $C_u$ and $C_s$ cells with age are present in the female data, although in the case of $C_s$ cells there are significant departures from linearity.

It is possible therefore that an increase in the number of cells with chromosome rearrangement with increasing age may be a general phenomenon. An increase in the number of aneuploid cells with age has already been reported [8, 9].

In the present study the effect of age alone is not sufficient to explain the observed results and it seems possible that we have been observing the different effects of a volatile agent on a range of people of varying ages. The present data is, however, not adequate for a detailed analysis of a possible interrelationship between these two factors.

Two important points about future studies of this kind arise from a consideration of the present investigation.

(1) It is obvious that the correct selection of controls is vitally important. Ideally each subject selected for study should be matched for age and sex with an individual working under conditions which are similar except for exposure to the possible chromosome damaging agent under consideration. When dealing with a volatile agent, such as benzene, it would be desirable also to introduce a second group of controls drawn from the population of subjects from which both the exposed and the control workers have been recruited.

(2) A study constructed in such a way would involve the counting and analysis of very large numbers of cells. If there is any possibility of the interaction of two independent variables, as in the present case, the numbers required for satisfactory statistical analysis would become quite unmanageable by the normal visual methods of chromosome analysis. This is the sort of problem which will be tackled much more easily when an automated system of

chromosome analysis such as those at present under development [10, 11] are available. Full development of these systems may take several years yet but in the very near future it may be able to discriminate between normal and abnormal cells [12]. When dealing with the effects of low doses of toxic agents where possibly more than 90% of the cells are normal, such a discrimination would give an enormous advantage. It is suggested that in the future, investigations of the type described in this paper will most satisfactorily be carried out using some form of automation of the procedure of chromosome analysis.

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## SUMMARY

*Chromosomes from the peripheral blood leucocytes of individuals exposed to benzene in the atmosphere have been examined. Three groups of factory workers were studied, and in two of these groups the percentage of cells with unstable chromosome aberrations* ($C_u$ *cells) was higher than would be expected from the general population. In one of the groups the increase in* $C_u$ *cells was confined to workers known to have been exposed to benzene, but in the other the controls and exposed workers both showed an increase in percentage of* $C_u$ *cells. The third group, who worked in a different type of factory, did not differ from the general population. In the two groups where an increase was seen the effect could have been due in part to environmental factors, but it is clear that the age of workers also influences the results and it is possible that an interplay between these two factors is responsible for the observed effect. It is suggested that automated chromosome analysis may play an important part in dealing with the large numbers of cells from exposed persons and controls which will be required in future studies of this type.*

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# Essai de Recherche d'Efficacité de l'Association de Prednisone, Vincristine, Méthyl-glyoxal-bis (guanylhydrazone) (Méthyl-GAG) et Méthotrexate sur les Leucémies Aiguës Monoblastiques

GROUPE COOPÉRATEUR DES LEUCÉMIES ET HÉMATOSARCOMES*
*l'Organisation Européenne de Recherche sur le Traitement du Cancer (O.E.R.T.C.)*

LE PRÉSENT article est destiné à rendre compte de l'efficacité d'une association de prednisone, vincristine, méthyl-glyoxal-bis (guanylhydrazone) (méthyl-GAG) et méthotrexate sur les leucémies aiguës monoblastiques.†

## MALADES TRAITES ET METHODES

(1) *Les malades*

Quinze malades atteints d'une leucémie aiguë monoblastique ont été l'objet de cet essai. Le diagnostic a été porté par un examen cytologique du sang et de la moelle osseuse. Etant donné les difficultés et les divergences de classification de telles leucémies, tous les frottis sanguins et de moelle osseuse des malades inclus dans cet essai ont été revus par un groupe de trois cytologistes. Les critères étaient ceux utilisés par G. Mathé et G. Seman [1]. On a exclu 7 malades pour lesquels on ne put obtenir l'accord d'au moins deux cytologistes sur le diagnostic de leucémie aiguë monoblastique.

L'âge des malades variait de 4 à 62 ans; on comptait 6 sujets du sexe féminin et 9 sujets du sexe masculin. Tous les malades étaient en phase visible de leur maladie, mais celle-ci pouvait être aussi bien la première que la deuxième phase.

(2) *Modalités d'administration de la chimiothérapie*

Le schéma thérapeutique était le suivant: prednisone 100 mg/m², tous les jours, par voie buccale; méthyl-GAG 150 mg/m², jour, pendant 3 jours de la semaine, par voie intraveineuse: méthotrexate 3 mg/m²/jour, les trois autres jours de la semaine, par voie buccale; vincristine 1 mg/m²/semaine (en une injection intraveineuse). La durée totale de cette chimiothérapie n'a pas été fixée à l'avance; elle a été soit la dose maximale tolérée, soit la dose suffisante pour obenir un effet optimal.

(3) *Surveillance*

Les malades étaient hospitalisés et soumis à des examens cliniques quotidiens, et hématologiques au moins bi-hebdomadaires.

*Membres ayant participé à cet essai:
*Hôtel-Dieu, Paris:* J. Bousser, G. Bilski-Pasquier, R. Zittoun, A. Bernadou, P. Blanc—*Institut de Cancérologie et d'Immunogénétique de l'Hôpital Paul-Brousse et Service d'Hématologie de l'Institut Gustave-Roussy, Villejuif:* G. Mathé, J. L. Amiel, A. Cattan, F. de Vassal, M. Hayat, Cl. Jasmin, J. R. Schlumberger, M. Schneider, L. Schwarzenberg—*Institut Jules Bordet, Bruxelles:* Y. Kenis—*Hôpital Edouard-Herriot, Lyon:* P. Croizat, L. Revol, J. J. Viala.

Les demandes de tirés à part doivent être adressées au Secrétaire: M. Hayat, Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse, 14, avenue Paul-Vaillant-Couturier, 94, Villejuif.

†"Leucémies aiguës monocytaires" de certaines nomenclatures.

*Tableau* 1. *Effets de l'association de prednisone, vincristine, méthyl-GAG, et méthotrexate sur les leucémies agiuës monoblastiques*

| | Rémissions complètes | Rémissions incomplètes | Echecs partiels | Echecs totaux |
|---|---|---|---|---|
| 1 ère phase visible | 2 | 3 | 1 | 5 |
| 2e phase visible | 1 | – | – | 3 |
| Total | 3 | 3 | 1 | 8 |

(4) *Appréciation des résultats*

Les résultats ont été appréciés à partir de deux types de symptômes: cliniques, en particulier l'hypertrophie des organes lymphoïdes, hypertrophie qui est, autant que possible, mesurée; hématologiques, par les frottis sanguins et de moelle osseuse.

L'essai ne comporte pas de sujets témoins: notre but, dans ce premier temps, n'est pas de comparer l'efficacité de cette association à celle d'un autre schéma thérapeutique, mais seulement de rechercher si elle est efficace. Nous considérons que l'on peut attribuer à notre thérapeutique un effet favorable observé, si les symptômes de la maladie s'aggravaient ou demeuraient stationnaires après une période-témoin préalable à l'essai.

## OBSERVATIONS ET ANALYSE DES RESULTATS

(1) *La tolérance*

Une aplasie myéloïde sévère a été observée dans 6 cas sur 15 malades traités, compliquée, pour l'un d'entre eux, d'une septicémie à pseudomonas, et, pour l'autre, d'une septicémie à staphylocoques dorés. Chez un autre malade, on a observé des complications neurologiques, notamment des convulsions, probablement dues à la vincristine.

(2) *Les résultats*

(a) *Résultats globaux.* Les résultats d'ensemble sont résumés au Tableau 1: sur 15 cures thérapeutiques, on compte 3 rémissions complètes, 3 rémissions incomplètes, 1 échec partiel et 8 échecs totaux.

(b) *Etude analytique des résultats.* Nous n'avons pas observé de différence d'effet selon l'âge et le sexe des patients. Les effets notables, c'est-à-dire les rémissions complètes et incomplètes, ont été enregistrés chez 5 malades qui étaient à la première phase visible de leur maladie, et chez 1 malade qui était à sa second phase, la première ayant été traitée par de la 6-mercaptopurine.

## DISCUSSION ET CONCLUSION

L'association essayée peut être considérée comme utile, puisque 3 patients sur 15 (soit environ 20%) ont bénéficié d'une rémission complète, et 3 autres d'une rémission incomplète. Elle peut donc être conseillée dans le traitement de la leucémie aiguë monoblastique.

Il n'est pas possible de comparer sa valeur à celle d'autres modalités thérapeutiques—composés divers administrés isolément, ou autres associations—étant donné l'extrême variabilité, selon les auteurs, des critères du diagnostic de cette affection.

On peut cependant admettre que le pourcentage des rémissions, dans les essais jusqu'ici publiés, était extrêmement bas [2], et que les résultats obtenus, dans cet essai, permettent de conseiller l'association de prednisone, vincristine, méthyl-GAG et méthotrexate comme traitement initial des leucémies aiguës monoblastiques.


## RESUME

*On a observé* 3 *rémissions apparemment complètes et* 3 *rémissions incomplètes chez* 15 *malades soumis à un essai de recherche d'efficacité de l'association prednisone, vincristine, méthyl-glyoxal-bis (guanylhydrazone) (méthyl-GAG) et méthotrexate sur la leucémie aiguë monoblastique.*

## SUMMARY

*Three apparently complete remissions and* 3 *incomplete remissions were obtained in* 15 *patients with the association of prednisone, vincristine, methyl-glyoxal-bis (guanylhydrazone) (methyl-GAG) and methotrexate in monoblastic acute leukemia.*

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# Analyse Caryotypique de Tumeurs Testiculaires

R. BERGER et M. MARTINEAU

*Maître de Recherche au C.N.R.S. Institut de Progénèse, 15 rue de l'Ecole de Médicine, Paris 6e, and*
*Department of Clinical Research, Institute of Cancer Research, Royal Marsden Hospital, London*

La diversité des anomalies, tant numériques que structurales, des chromosomes des cellules tumorales, leur variabilité d'une cellule à une autre, dans une même tumeur et d'une tumeur à une autre, rendent difficile l'analyse des modifications chromosomiques observées dans les cancers.

Il apparaît cependant que la comparaison des anomalies existant dans des tumeurs de même type est nécessaire si l'on veut tenter d'établir des corrélations entre les caractéristiques histologiques, l'évolutivité ou la réponse à la thérapeutique. Outre l'impossibilité où nous sommes de reconnaître individuellement de nombreux chromosomes, et notre ignorance portant sur le contenu génique des chromosomes reconnus sur leur seule morphologie, les deux obstacles majeurs aux comparaisons nécessaires restent la variabilité du nombre des chromosomes et la présence d'éléments remaniés habituellement désignés sous le terme de marqueurs. La comparaison des chromosomes marqueurs entre eux a été tentée, à plusieurs reprises, par les méthodes statistiques habituelles ou avec la méthode dite du flou statistique [1–3], et nous ne les détaillerons pas ici. Les résultats de ces études ont été variables, tantôt l'identité des marqueurs paraît vraisemblable, tantôt on peut conclure que les marqueurs sont différents. En tout état de cause, il ne faut pas perdre de vue que la comparaison porte seulement sur la morphologie et qu'on ne peut jamais affirmer qu'une ressemblance morphologique corresponde à une identité génétique.

La variété du nombre des chromosomes dans les différentes tumeurs apporte également un élément de complexité car il devient difficile de comparer la distribution des chromosomes dans chaque groupe de la classification internationale pour des tumeurs différentes. C'est la raison pour laquelle la méthode dite des profils a été mise au point [3] qui vise à rassembler, dans une seule représentation, la quantité maximum d'informations, permettant ainsi une estimation rapide du degré de ressemblance ou de dissemblance des tumeurs étudiées. Nous avons appliqué cette méthode aux résultats de l'étude chromosomique d'une série de tumeurs testiculaires humaines [2, 4].

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## MATERIEL ET METHODES

Les détails concernant la technique et le résultat de l'examen cytogénétique des tumeurs testiculaires a fait l'objet de rapports précédents [2,4]. Le matériel, étudié essentiellement grâce à une méthode directe de préparation, est constitué de dix séminomes (deux d'entre eux étant apparus chez le même malade), sept tératomes malins et six tumeurs combinées (tératomes et séminomes). L'une de ces dernières (R.M.) avait d'abord été considérée comme un séminome lors du premier examen histologique et classée comme tel dans le premier ouvrage cité.

La méthode dite des profils a pour but de résumer, en une seule représentation, le maximum d'informations sur les caryotypes obtenus. Elle consiste à obtenir une image composite, grâce à la superposition photographique des "profils" individuels de chaque cellule. Ces profils individuels sont obtenus en représentant chaque groupe de chromosomes (ou chaque paire de chromosomes individuellement reconnaissables) sur des axes en coordonnées polaires, la longueur des abscisses étant choisie en fonction de la longueur relative des

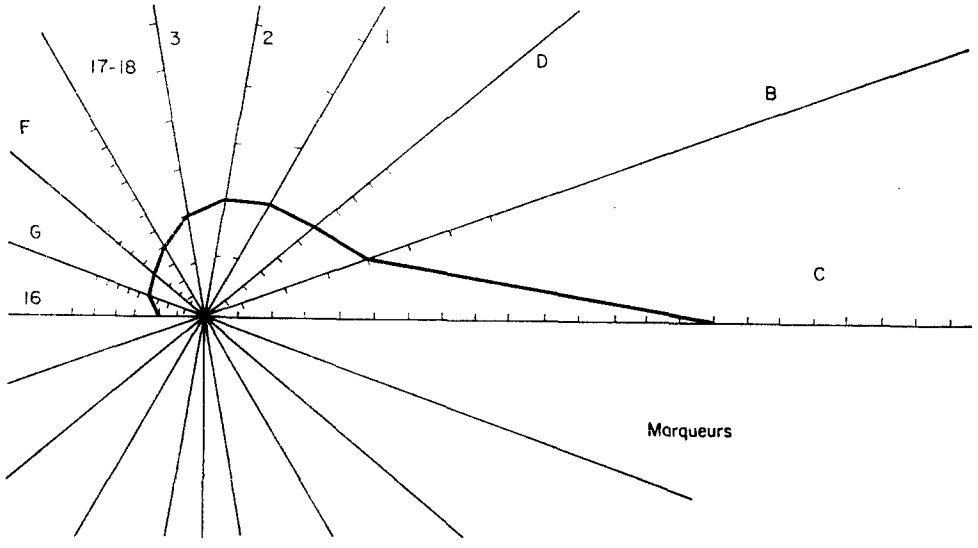



*Fig. 1. Méthode des profils. Choix des axes en coordonnées polaires. Le tracé du caryotype de l'homme normal a été représenté.*

chromosomes représentés. Les points extrêmes sont reliés entre eux, déterminant le "profil" de chaque cellule individuelle. Le choix des axes a été établi de telle sorte que le profil de la cellule normale soit aussi régulier que possible, et l'on a placé de droite à gauche les axes correspondant aux chromosomes C, B, D, 1, 2, 3, 17–18, F, G, 16 (Fig. 1); les chromosomes marqueurs sont représentés au-dessous de l'axe horizontal. Chaque profil est photographié. L'analyse peut se faire alors soit en "grandeurs réelles," soit en "grandeurs relatives".

—En grandeurs réelles le rapport d'agrandissement choisi est constant pour toutes les cellules. Le négatif du profil de chaque cellule est projeté successivement sur une plaque photographique en faisant coïncider les axes des coordonnées. On obtient ainsi la superposition de profils de dimensions variées.

—En grandeurs relatives, le rapport d'agrandissement est modifié pour chaque cellule en tenant compte de la longueur du matériel chromosomique, soit L. La longueur L est obtenue en additionnant les longeurs relatives individuelles de chaque chromosome, y compris les chromosomes marqueurs. Si D est le rapport d'agrandissement choisi pour une cellule normale, le rapport d'agrandissement pour la cellule tumorale est donnée par la formule: $1968 \times D/L$ (1968 représentant la longueur du matériel chromosomique du caryotype de l'homme normal, celui de la femme étant par définition 2 000). L'avantage des coordonnées polaires est que malgré les modifications apportées par les variations des rapports d'agrandissement, on peut faire coïncider exactement les axes des coordonnées. Les profils alors obtenus sont directement comparables les uns aux autres, ainsi qu'avec le profil normal de sexe correspondant.

*Les méthodes d'étude numériques* utilisables peuvent être diverses. L'une des méthodes consiste à calculer le nombre moyen de chromosomes de chaque groupe de la classification internationale en ramenant le nombre de chromosomes moyen par cellule tumorale à la normale; ou, au contraire, à comparer le nombre de chromosomes de chaque groupe à la valeur théorique obtenue à partir du caryotype normal mais ramené au nombre total de chromosomes moyens par cellule, observé dans la tumeur. Ces méthodes reviennent en somme à calculer la distribution dans chaque groupe, en tenant compte du degré de ploïdie des cellules tumorales. Il reste à comparer, en général avec les méthodes statistiques habituelles, les valeurs observées et les valeurs théoriques [5–8]. Ces méthodes, ou celle un peu différente de Sandberg et coll. [9], ont l'inconvénient de laisser une ambiguïté quant à la place des chromosomes marqueurs. De plus elles obligent à comparer des répartitions de chromosomes dans des cellules cancéreuses et des cellules normales, les artefacts (perte de chromosomes surtout) étant sans doute d'amplitude très différente dans les deux sortes de cellules. L'expérience montre en effet que les réactions au choc hypotonique, utilisé lors de la préparation des lames microscopiques, sont en général très différentes pour les cellules normales et pour les cellules tumorales.

La méthode dite des rapports vise à étudier un autre aspect de la répartition des chromosomes de chaque groupe: elle consiste à apprécier le rapport entre les nombres chromosomiques observés dans chaque groupe de

chromosomes deux à deux (1 à 2, 1 à 3, etc ... F à G). La déviation de ce rapport d'avec la normale donne une idée des modifications de dosage génique dans les cellules tumorales. Là encore, la comparaison statistique avec la normale est rendue difficile du fait des réactions différentes des cellules aux techniques utilisées. Si les pertes de chromosomes se font en grande partie au hasard, on minimise les variations accidentelles en prenant, pour chaque groupe de chromosomes, les nombres totaux et non les rapports de chaque cellule prise individuellement.

## RESULTATS ET DISCUSSION

*Méthode des profils*

Les profils "en grandeurs relatives", obtenus à partir de l'étude des chromosomes des tumeurs testiculaires, sont reproduits dans les Fig. 2, 3 et 4. Certaines constatations peuvent être faites à partir de leur examen:

(1) le degré d'hétérogénéité des carytotypes, c'est-à-dire la coexistence, dans la même tumeur, de cellules possédant des caryotypes différant les uns des autres, peut être appréciée sur la présence de dégradés au pourtour des profils. D'une manière générale, ces tumeurs sont hétérogènes, mais d'une manière inégale. En moyenne, les séminomes paraissant moins hétérogènes que les tumeurs combinées, elles-mêmes plus homogènes que les tératomes malins (aucun tératome n'est homogène alors que 2 tumeurs combinées sur 6, et 4 séminomes sur 9, le sont relativement).

(2) La comparaison des profils obtenus, pour une même tumeur, à partir des cellules examinées directement et de celles qui ont été cultivées, a été faite dans deux cas. Dans une tumeur combinée (HB, examen direct et culture de 3–4 jours) malgré des différences, les profils sont très comparables.

L'examen portant sur un petit nombre de caryotypes (28 pour l'examen direct et 16 pour la culture), il est difficile de savoir si les différences observées sont dues à la sélection *in vitro*, à la variation aléatoire, ou au fait que les fragments de tumeur, examinés directement et cultivés, étaient réellement différents *in vivo*.

Dans le cas du tératome DH (examen direct et culture de 4–6 jours) des différences importantes peuvent être notées entre les deux profils obtenus. L'effet d'une sélection différentielle *in vitro* selon le caryotype des cellules ne pouvant être écarté, il paraît préférable de comparer des tumeurs examinées directement, lorsque l'on désire comparer les anomalies chromosomiques de différentes tumeurs.

(3) Dans le cas de séminome bilatéral ($EB_L$ et $EB_R$) il est difficile de savoir s'il s'agit ou non de la même tumeur, c'est-à-dire de savoir si les deux tumeurs ont une origine indépendante, ou si l'une est une métast ase de l'autre. La question ne peut être résolue par le seul examen cytogénétique, puisque l'on connaît des exemples pour lesquels la tumeur primitive et les métastases avaient des caractéristiques chromosomiques différentes [3].

Cependant, on peut remarquer ici que le nombre modal des chromosomes est différent selon la tumeur considérée, que les marqueurs sont différents, et que les profils, quoique apparentés, offrent des dissemblances.

(4) Une classification des tumeurs, à partir des profils, est proposée ci-dessous. Sont classées sur une même colonne verticale les tumeurs dont les profils présentent des analogies communes, et sur une ligne horizontale ceux qui paraissent différents, l'ordre proposé étant celui de leur éventuelle ressemblance. On a ainsi:

| | | | | | |
|---|---|---|---|---|---|
| Séminomes: | DR | GK | KD | JP | PH |
| | $EB_R$ | PC | | | |
| | $EB_L$ | | | | |
| | JC | | | | |
| | NH | | | | |
| Tumeurs combinées: | HB | BR | CW | | |
| | SH | | | | |
| | AH | | | | |
| | RM | | | | |
| Tératomes malins: | BG | VR | TB | JB | SM MJ |
| | DH direct | | | | |
| | DH culture | | | | |
| | (le profil de MJ ressemble un peu à ceux du groupe majoritaire des séminomes). | | | | |

L'aspect particulier des séminomes est dû, en particulier, au contraste entre l'excès de chromosomes du groupe F et le déficit en 17–18 apparent sur le profil composite.

On ne parvient pas à classer à l'aveugle, de façon stricte, les tumeurs en trois catégories. Cependant, le premier groupe des séminomes (DR, EB etc...) présente de réelles ressemblances. La comparaison du classement de ces tumeurs à partir des profils d'une part, et à partir des données anatomo-pathologiques d'autre part, n'a pu encore être faite.

*Méthodes numériques*

(1) Nombres chromosomiques dans les différents groupes. En ramenant au nombre théorique normal de 46 le nombre moyen de chromosomes, observé par cellule tumorale (marqueurs inclus), on obtient les nombres

moyens de chromosomes pour chaque groupe de la classification internationale. En tenant compte des valeurs différant de plus de 10% du nombre normal dans chaque groupe (soit 0,20 pour les chromosomes 1, 2, 3 et 16, 0,40 pour les chromosomes des groupes B, 17–18, F, etc. . . .), on a les déviations suivantes:

Dans tous les séminomes, on observe un déficit dans les groupe B; dans 5 sur 9 ou 10 (selon que l'on estime que $EB_R$ et $EB_L$ sont ou non une même tumeur) un déficit dans le groupe D, et dans 6 sur 9 ou 10 dans le groupe 17–18. Un excès de chromosomes 16 est observé dans 4 tumeurs, d'éléments du groupe F dans 5 tumeurs, et du groupe G dans 4 tumeurs.

Pour les tumeurs combinées, on constate un déficit dans le groupe B dans 5 cas sur 6, dans le groupe D dans 3 cas, un excès de F dans 5 cas, de n° 16 dans 3, et de n° 2 dans 3 cas.

Pour les tératomes malins enfin, un déficit en n° 1 dans 4 cas sur 7, en B dans 7 cas sur 7, en D dans 4 cas sur 7 et un excès de n° 16 dans 4 cas sur 7 ont été relevés.

Les points communs sont donc la perte de chromosomes du groupe B et la tendance à la perte de chromosomes du groupe D. Les différences consistent en la tendance à l'excès de chromosomes F dans les séminomes et les tumeurs combinées, en déficit en éléments du groupe 17–18 dans les séminomes, et en la tendance en déficit en 17–18 des tumeurs combinées. Ces deux modifications ne sont pas retrouvées dans les tératomes malins.

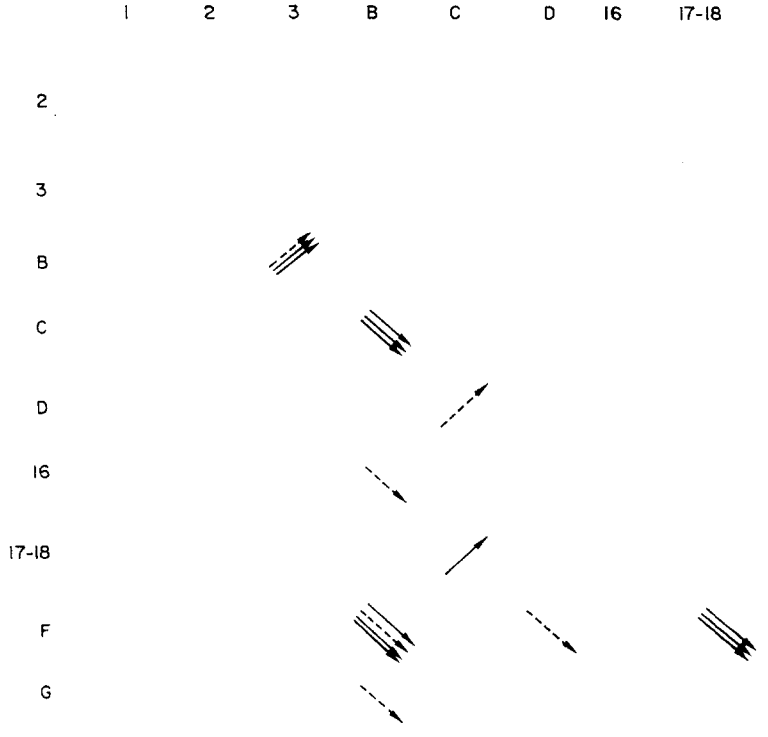
L'excès relatif systématique de chromosomes du groupe C et la perte d'éléments du groupe G, signalés par Levan et van Steenis, n'ont pas été retrouvés dans l'ensemble de ces tumeurs testiculaires. Par contre, la tendance au déficit en chromosomes des groupes B et D a été signalée dans d'autres types de tumeurs, comme les cancers ovariens, les mélanomes malins, et les tumeurs digestives [3].

*Etude des rapports de groupe à groupe*

La grande majorité des rapports évalués varie en plus ou en moins par rapport à la valeur théorique normale. Si l'on ne tient compte, pour chaque rapport, que des variations qui se font dans le même sens pour chacune des tumeurs prise individuellement, on constate (tableau 1) que les rapports du nombre de chromosomes du groupe B au nombre du chromosomes du groupe C (B/C), les rapports B/F et 17–18, sont diminués et le rapport C/17–18 augmenté dans tous les séminomes. Les rapports B/16, B/F, B/G et B/G et D/F sont diminués dans les tératomes, et les rapports 3/B et C/D augmentés.

Enfin les rapports B/C, et 17–18/F sont diminués dans les tumeurs combinées comme dans les séminomes; le rapport 3/B est augmenté et le rapport B/F y est diminué comme dans les tératomes. Il est remarquable que les

*Tableau* 1. *Déviations concordantes des rapports des nombres de chromososes de groupe a groupe: numérateur sur les lignes horizontales, dénominateur sur les colonnes verticales. Le sens de la flèche indique le sens de la déviation. Séminomes en traits pleins, tératomes en pointillés, tumeurs combinées en traits doubles.*



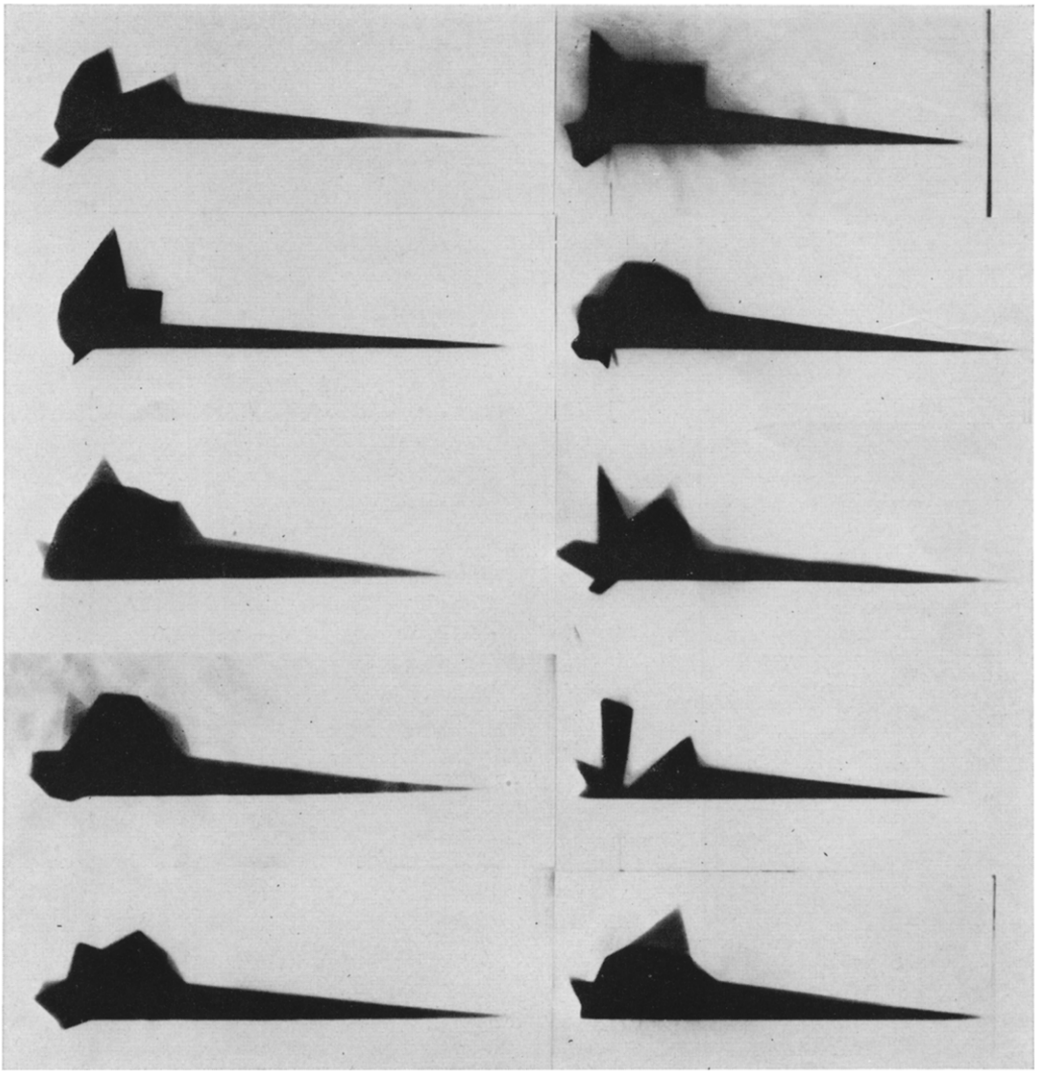


*Fig. 2.* *"Profils en grandeurs relatives" des séminomes. De haut en bas, colonne de gauche: JP, PH, JC, $EB_L$, $EB_R$; colonne de droite: PC, DR, GK, KD et NH (marqueurs non représentés).*

(to face p. 64)

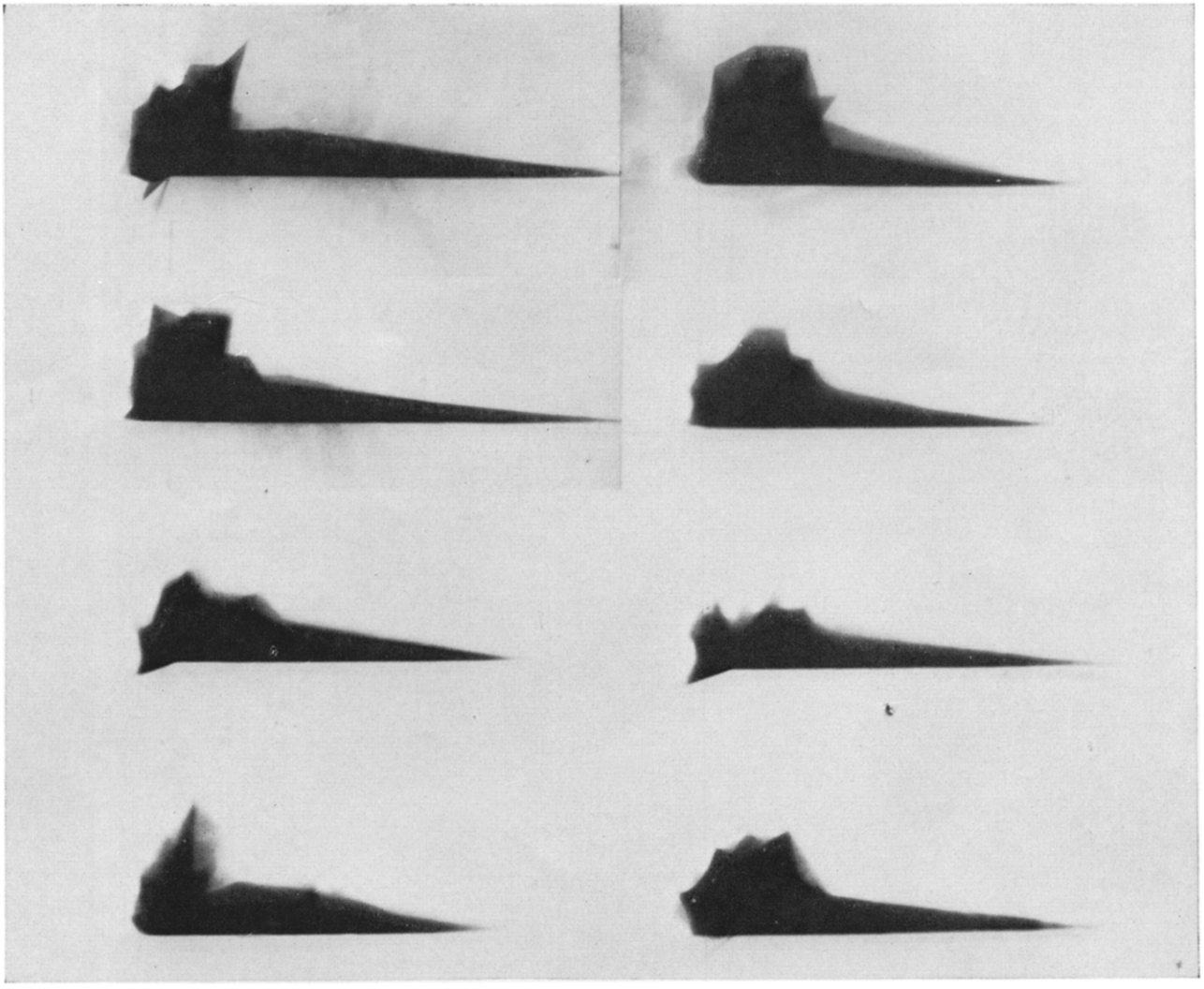


*Fig.* 3. "*Profils en grandeurs relatives*" *des tératomes malins. De haut en bas, colonne de gauche: DH (culture), DH (direct), JB, TB (marqueurs non représentés); colonne de droite: BG, VR (marqueurs non représentés), SM MJ.*

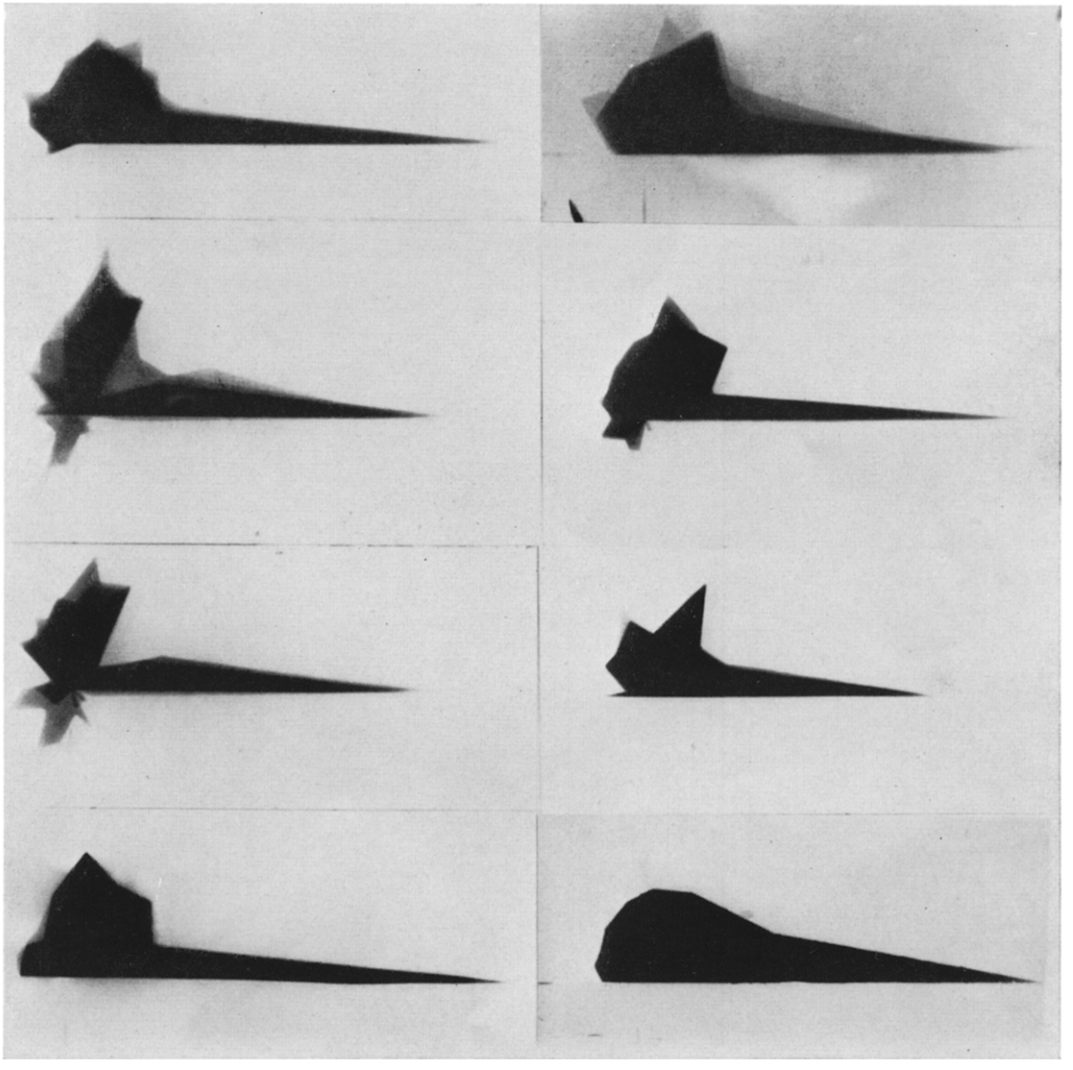


*Fig. 4. "Profils en grandeurs relatives" des tumeurs combinées. De haut en bas, colonne de gauche: SH, HB (direct), HB (culture), RM (marqueurs non représentés); colonne de droite: BR (marqueurs non représentés), AH, CW (marqueurs non représentés), homme normal à la même échelle.*

variations des rapports du nombre de chromosomes de groupe à groupe dans les tumeurs combinées se fassent partiellement à la fois comme dans les tératomes et comme dans les séminomes. Les variations de ces rapports sont par ailleurs différentes (si l'on tient compte des variations concordantes dans une série de tumeurs comparables) de celles observées dans des tumeurs d'autre origine, telles que cancers ovariens, recto-coliques et mélanomes malins [3]. Il reste malheureusement difficile d'évaluer statistiquement la signification de la déviation des rapports d'avec leur valeur théorique normale et il faut remarqueur que sur 45 rapports calculés, un certain nombre d'entre eux doivent avoir une variation concordante du seul fait du hasard. Cependant, le fait que les tumeurs combinées paraissent, à ce point de vue, intermédiaires entre séminomes et tératomes malins paraît particulièrement intéressant.

## CONCLUSIONS

Le nombre de tumeurs testiculaires analysées dans la présente étude est trop restreint pour que l'on puisse tirer des conclusions générales ou définitives. Il apparaît cependant que les anomalies chromosomiques constatées varient dans une large mesure d'une tumeur à une autre, ainsi que de nombreux auteurs l'ont déjà constaté. Les résultats obtenus avec les méthodes employées (méthode des profils, méthode des rapports en particulier) sont très différents de ceux obtenus dans l'étude des mélanomes malins, des cancers recto-coliques et ovariens, faite avec les mêmes méthodes. Ce type d'analyse doit être poursuivi si l'on veut tenter d'établir des corrélations entre anomalies chromosomiques et données anatomo-cliniques. Seule une analyse patiente et détaillée de nombreuses tumeurs permettra de mieux comprendre la signification des anomalies chromosomiques des tumeurs.

**Remerciements**—Nous remercions très vivement les Docteurs S. D. Lawler, E. N. K. Wallace, P. E. Thompson Hancock et le "Fellowship Committee of the Royal Marsden Hospital" pour le "Gordon Jacobs Fellowship."

## RESUME

*De nouvelles méthodes d'analyse des chromosomes ont été appliquées à l'étude des tumeurs testiculaires humaines, séminomes, tératomes malins et tumeurs combinées (séminomes et tératomes), dans le but de comparer les anomalies observées dans ces différentes tumeurs. La méthode dite des profils, qui vise à résumer en une seule représentation composite le maximum d'informations sur les caryotypes obtenus, a montré des analogies, mais aussi des différences, entre les tumeurs examinées. Un aspect particulier des séminomes, dû à l'excès des chromosomes du groupe F et au déficit en chromosomes* 17–18, *a été mis en évidence. Un classement des tumeurs, à partir des données cytogénétiques, a été proposé, mais il n'a pas été possible de distinguer formellement trois groupes distincts correspondant aux trois types de tumeurs. La méthode des rapports, qui consiste à évaluer les rapports du nombre de chromosomes de chaque groupe de la classification deux à deux, a permis de constater certaines déviations concordantes pour toutes les tumeurs de chaque groupe (rapports B/C, B/F, et* 17–18/*F diminués et rapport C/*17–18 *augmenté dans les séminomes; rapports B/*16. *B/F, B/G et D/F diminués et* 3/*B et C/D augmentés dans les tératomes malins; rapports B/C, B/F,* 17–18/*F diminués et* 3/*B augmenté dans les tumeurs combinées). Le fait que certaines des déviations d'avec la normale, rencontrées dans les tumeurs combinées, soient communes à la fois aux séminomes et aux tératomes semble particulièrement remarquable.*

## SUMMARY

*New methods for chromosome analysis were applied to the study of testicular tumours, seminomas, malignant teratomas and combined tumours (seminoma+malignant teratoma) in order to compare the abnormalities observed in these tumours.*

*The profile method, which was intended to summarize all the karyotypes of a tumour in a single composite image, showed consistent and divergent features between the tumours. Seminomas had a characteristic profile which reflected an excess of F-group chromosomes and a lack of Nos.* 17–18. *The tumours were classified by ther profiles, but it was not possible in this way clearly to distinguish the three histological types.*

*The proportional method, in which were evaluated the ratios between the complements of the chromosome groups taken in pairs, showed that certain deviations from the normal were consistent throughout a histological type (seminomas-proportions of B/C, B/F,*

*17–18/F, decreased and C/17–18 increased; malignant teratomas—B/16, B/F B/G, D/F decreased and 3/B, C/D increased; combined tumours—B/C, B/F 17–18/F decreased and 3/B increased). It seems noteworthy that the deviations recorded for the combined tumours were common to either or both of the other two types.*

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# The Biochemical Mechanism of Selective Heat Sensitivity of Cancer Cells

## III. Studies on Lysosomes

C. TURANO, A. FERRARO, R. STROM, R. CAVALIERE and A. ROSSI FANELLI

*Institute of Biological Chemistry, University of Rome, Italy, and Regina Elena Institute for Cancer Research, Rome, Italy*

BIOCHEMICAL and clinical evidence has recently been presented that neoplastic cells are more sensitive than normal cells to high temperature [1, 2]. Oxygen consumption, as well as incorporation of radioactive precursors into nucleic acids and proteins, are inhibited by exposure of neoplastic cells to 42–43°C [1, 3, 4]. Since lysosomes, with their content of hydrolytic enzymes, are capable of carrying out degradative processes on most cellular components, and possibly on the whole cellular structure [5], it seemed interesting to compare their thermal stability in normal, regenerating and neoplastic cells.

Since the properties of lysosomes from different tissues are known to vary widely [6], similar types of cells had to be compared. Novikoff hepatoma was used as the source of tumor cells, allowing a valid comparison to be made either with normal rat liver hepatocytes, or, to compensate for the higher number of mitoses, with regenerating rat liver cells. In a few experiments, however, mouse Ehrlich ascites tumor cells, which have the advantage of a greater homogeneity of population, were used.

The possibility that lysosomes are the cause of the specific sensitivity of tumor cells to high temperatures was also investigated on whole cells by measuring the effect, on cellular respiration and on heat sensitivity of the cells, of agents and/or procedures known to either protect or disrupt specifically the lysosomes within the cell itself.

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### MATERIAL AND METHODS

Male Sprague Dawley rats were used as the source of normal liver, of regenerating liver, which was taken 24 hr after partial hepatectomy, and of Novikoff hepatoma, which was removed six days after intraperitoneal transplantation. Cell suspensions were prepared as previously described [1]. For the isolation of lysosomes the cells, suspended in 0·4 M sucrose, were briefly homogenized at 1200 rev/min in a Potter–Elvehjem apparatus. One minute homogenization was sufficient, with these cells, to give a satisfactory yield of lysosomes [7].

Ehrlich ascites tumor cells were grown i.p. in Swiss mice, and collected 7–8 days after inoculation. Hemorrhagic suspensions were discarded. These cells were found to be resistant to the homogenization under the conditions described above, and were disrupted by sonication, in 0·4 M sucrose, in a MSE 100 watt ultrasonic disintegrator for 3 sec.

The suspension obtained by either of these methods was centrifuged at 800 **g** for 10 min, to sediment unbroken cells and nuclei. The

supernatants were then incubated at 37 or 43°C for different times; the pH remained constant in all cases during the incubation. At chosen intervals aliquots were withdrawn, cooled immediately in an ice bath and centrifuged at 16,000 **g** for 20 min. The pellet, containing lysosomes and mitochondria, was suspended in distilled water, frozen and thawed three times, and analysed for the following enzymes: DNAase, RNAase, acid phosphatase and cathepsins. Enzymatic activities measured after the described procedure are assumed to represent the activities derived from intact lysosomes, i.e. from lysosomes that survived incubation at 37 or 43°C; the enzymes from damaged lysosomes are instead discarded with the supernatant of the last centrifugation. Adsorption of enzymes on the pellet was not taken into consideration, since it would not be expected to vary largely as a consequence of previous incubation in the conditions described. This measure of the residual lysosomes has the advantage that the activity of enzymes freed during the homogenization procedure does not interfere in the determinations. This is particularly important when the Novikoff hepatoma is being studied, since its lysosomes show an increased mechanical lability and rather large fractions of acid hydrolases are found in the supernatant. Assay of enzymes in the supernatant did not show any appreciable inactivation during the incubation, the pattern obtained matching more or less precisely the one followed by the enzymes in the residual lysosomes.

The following methods have been used for activity determinations: DNAase and RNAase were determined according to De Duve *et al.* [7]; catheptic activity according to Press, with denatured hemoglobin as substrate [8]; acid phosphatase was determined using p-nitrophenylphosphate as substrate [9].

For the experiments with whole cells, a Novikoff cell suspension in Krebs–Ringer-phosphate, as previously described [1], was used. Oxygen consumption was measured in a conventional Warburg apparatus.

Intracellular photolysis of lysosomes was performed with a Sylvania 4-DCA tungsten lamp, 150 W, 21·5 V, with incorporated parabolic mirror. The cell suspension was contained in a silver-plated 600 ml beaker, 30 cm from the lamp. Temperature control was ensured by filtering the light beam through an antithermic glass and a 2 cm layer of water, and by cooling the beaker with ice. The final temperature of the cell suspension was 8–10°C.

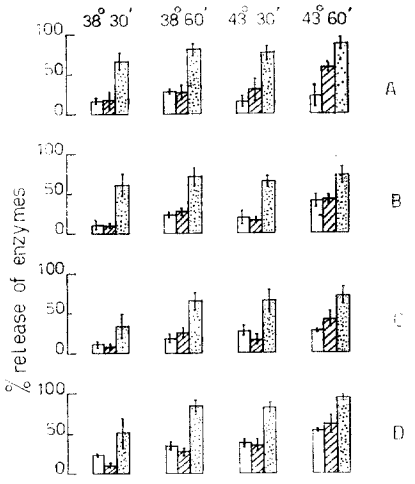


*Fig.* 1. *Percentage release of enzymes from lysosomes of normal liver, regenerating liver and Novikoff hepatoma cells, at* 38° *and* 43°*C.*

*The enzymes released after incubation of lysosomes for* 30 *min or* 60 *min at* 38 *or* 43°*C in* 0·4 M *sucrose are expressed as percentage of the total enzymatic activity which may be released by repeated freezing and thawing in distilled water. The enzymatic activities investigated were: RNAase* (*A*), *cathepsins* (*B*), *acid phosphatase* (*C*) *and DNAase* (*D*).

*Lysosomes from normal liver* (□), *regenerating liver* ( ▨ ) *and Novikoff hepatoma* ( ▦ ) *were used. Each result is the mean of* 3 *or* 4 *determinations. Standard deviations are shown by vertical lines.*

## RESULTS

### *Effect of high temperature on lysosomes from normal, regenerating and neoplastic cells*

As seen from Table 1, where the results of a typical experiment are reported, and from Fig. 1, which shows the behaviour of various enzymes from different cells, lysosomes of Novikoff hepatoma appear to be, in a general way, significantly more labile.

It is interesting to note that, while in the normal or regenerating liver all the enzymes considered are released from the lysosomes at similar rates, as could be expected from the simplest hypothesis of a uniform lysosomal population, in Novikoff hepatoma the rates are different for the various enzymes: leakage of RNAase and of the cathepsins occurs mostly during the first 30 min, and there is no appreciable difference between 38 and 43°C (Fig. 1, A and B). Liberation of acid phosphatase and DNAase is instead less prompt, and is greatly enhanced by raising the temperature (Fig. 2, C and D).

In Ehrlich ascites tumor cells, also, release of acid phosphatase follows a pattern quite distinct from that of cathepsins, the difference being of the same type as that observed in Novikoff hepatoma cells.

*Table* 1. *Release of hydrolytic enzymes from lysosomes of normal rat liver, regenerating liver and Novikoff hepatoma cells*

*Activities, expressed as enzyme units (as defined by Ref. 7–9) per gram of wet tissue, were measured after isolation of the residual lysosomes and complete unmasking of their enzymes by freezing and thawing in $H_2O$.*

| Incubation time and temperature (°C) of the lysosome containing fraction | Normal liver | | Regenerating liver | | Novikoff hepatoma | |
|---|---|---|---|---|---|---|
| | Enz. un./g tissue | % release | Enz. un./g tissue | % release | Enz. un./g tissue | % release |
| RNAase | | | | | | |
| 0 min | 0·676 | — | 0·330 | — | 0·990 | — |
| 30 min, 38° | 0·545 | 19 | 0·260 | 21 | 0·090 | 91 |
| 60 min, 38° | 0·515 | 24 | 0·210 | 36 | 0·082 | 92 |
| 30 min, 43° | 0·470 | 31 | 0·238 | 28 | 0·060 | 94 |
| 60 min, 43° | 0·440 | 35 | 0·141 | 58 | 0·053 | 95 |
| Cathepsines | | | | | | |
| 0 min | 0·683 | — | 0·465 | — | 0·286 | — |
| 30 min, 38° | 0·620 | 9·2 | 0·398 | 15 | 0·077 | 73 |
| 60 min, 38° | 0·533 | 22 | 0·348 | 25 | 0·066 | 77 |
| 30 min, 43° | 0·485 | 29 | 0·364 | 22 | 0·064 | 78 |
| 60 min, 43° | 0·383 | 44 | 0·330 | 29 | 0·057 | 80 |
| Acid Phosphatase | | | | | | |
| 0 min | 1·45 | — | 0·595 | — | 0·455 | — |
| 30 min, 38° | 1·33 | 8·3 | 0·524 | 12 | 0·241 | 47 |
| 60 min, 38° | 1·23 | 15 | 0·416 | 30 | 0·115 | 75 |
| 30 min, 43° | 1·03 | 29 | 0·399 | 33 | 0·088 | 81 |
| 60 min, 43° | 0·90 | 38 | 0·323 | 46 | 0·072 | 84 |
| DNAase | | | | | | |
| 0 min | 0·338 | — | 0·164 | — | 0·033 | — |
| 30 min, 38° | 0·264 | 22 | 0·146 | 11 | 0·022 | 33 |
| 60 min, 38° | 0·226 | 33 | 0·110 | 33 | 0·007 | 79 |
| 30 min, 43° | 0·240 | 29 | 0·107 | 35 | 0·008 | 76 |
| 60 min, 43° | 0·162 | 52 | 0·066 | 60 | 0·003 | 91 |

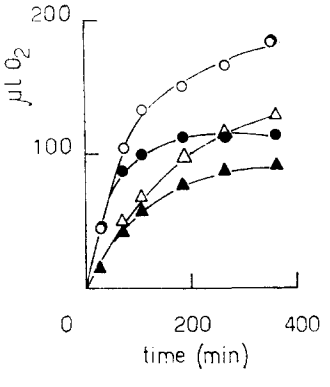


*Fig.* 2. *Effect of exposure to light on $O_2$ consumption of neutral red treated Novikoff hepatoma cells.*

*$O_2$ consumption at 38°C (○, △) and 43°C (●, ▲) by Novikoff hepatoma cells which had been exposed for 90 min to light (△, ▲) after incubation with neutral red, measured using glucose + succinate as substrate. Controls (○, ●) were performed with cells preincubated with the dye but protected from the action of light.*

*Effect of intracellular lysosomal damage upon oxygen consumption by hepatoma cells*

According to Allison [10], many dyes are concentrated into lysosomes. Some of these dyes transfer to the membranes of these organelles the energy absorbed from appropriate electromagnetic radiations, acting therefore as photosensitizers. Since by such means the cellular damage starts at the lysosomal level [10, 11], it was checked whether this procedure can reproduce the effects of heat on cellular respiration.

Novikoff hepatoma cells were therefore, after washing with Krebs–Ringer-phosphate, incubated with 1 mg/100 ml of neutral red. After 1 hr at 38°C in the dark, the cells were washed three times, then exposed for 90 min to visible light, as described under Methods. Neutral red has, at neutral pH, an absorption maximum at 460 nm and a shoulder around 525 nm; the tungsten lamp used has its maximal emission between 400 and 600 nm. Controls were made with cells pretreated with neutral red but kept in the dark.

The cell suspensions were subsequently assayed for $O_2$ consumption in the presence of

0·015 M glucose+0·013 M succinate. As can be seen from Fig. 2, $O_2$ consumption by photoirradiated cells is decreased. Nevertheless, a raise in temperature from 38 to 43°C, which has a striking inhibitory effect on respiration of non-irradiated cells, causes a further inhibition also of the irradiated ones. In both cases the lag-time in the onset of inhibition is similar—around 60 min.

*Effect of inhibition of lysosomal enzymes upon oxygen consumption by hepatoma cells*

Trypan blue is another dye which is preferentially incorporated into lysosomes [10]. At the microscope, Novikoff hepatoma cells incubated with this dye, show after a few minutes, several blue granules scattered throughout the cytoplasm: such cells are anyhow viable, since the picture is quite different from the diffuse staining which characterizes [12] dead cells.

According to Lloyd *et al.* [13–15], trypan blue has an inhibitory action both upon the release and the activities of the hydrolytic enzymes contained in the lysosomes. We have therefore checked whether the addition of trypan blue influences the inhibitory effect of heat upon respiration of hepatoma cells. As can be seen from Fig. 3, the presence of 2 mg/ml of this dye in the incubation mixture does not make Novikoff hepatoma cells resistant to the higher temperature. It may be recalled that this concentration inhibits almost completely the lysosomal enzymes [13] and, as may be seen also from Fig. 3, has probably a slight inhibitory effect also on enzymes of the respiratory pathway, although it does not affect cell viability [12]. At a cencentration of 15 mg/ml respiration is decreased by more than 50%. Anyhow, in no case is the onset or the magnitude of the inhibition by heat appreciably modified. In fact, in the presence of 2 mg/ml trypan blue, the oxygen consumption at 38°C remains linear for a somewhat longer time than in its absence — this effect may be due to such an inactivation of the hydrolytic enzymes.

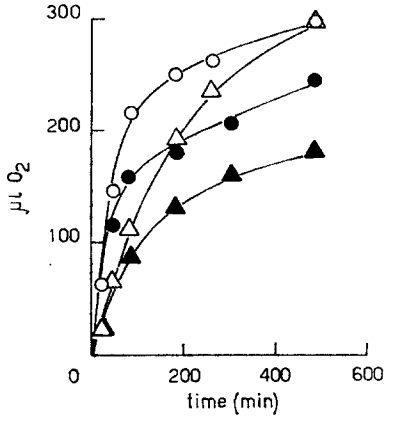


*Fig. 3. $O_2$ consumption of Novikoff hepatoma cells in the presence of trypan blue.*
*$O_2$ consumption by Novikoff hepatoma cells was measured at 38°C (○, △) and 43°C (●, ▲), with glucose and succinate as substrates, in the presence (△, ▲) or absence (○, ●) of 2 mg/ml of trypan blue.*

## DISCUSSION

Isolated lysosomes from both Novikoff hepatoma and Ehrlich ascites tumor cells show a striking lability already at 38°C. An increased labilization by higher temperatures, such as previous work had evidenced in neoplastic cells at the respiratory and biosynthetic levels [1–4] can be shown only on the release of lysosomal acid phosphatase and DNAase. This non-homogeneity of behaviour between the various enzymes, which is absent from lysosomes from normal and regenerating liver, may be a peculiarity of neoplastic cells. It is indeed possible that some or even most of the lysosomes in a Novikoff hepatoma suspension originate from non-neoplastic cells (i.e. fibroblasts, macrophages, granulocytes), since neoplastic cells seem to have fewer of these organelles, which may moreover have a higher mechanical fragility.

However, lysosomes from a homogeneous tumor cell population, such as Ehrlich ascites cells, also show enzymatic heterogeneity. Horvat and Touster [16] had already noticed, in Ehrlich ascites tumor cells, this peculiarity. Nevertheless, in their experiments, a certain fraction of lysosomal activities was not released after long incubation at 37°C, and the authors concluded that the thermal resistance of lysosomes from tumor cells is greater than that of lysosomes in normal liver. In our hands, using shorter incubation times and, when indicated, higher temperatures, lysosomes from neoplastic cells were found to be considerably more fragile than those from normal or regenerating liver. The heterogeneity in the lysosomal population was evidenced, in our experiments, by the different rate of release of the various enzymes.

The question whether this lysosomal fragility can fully explain the peculiar sensitivity of neoplastic cells to elevated temperatures [1, 4], is still unresolved. The experiments with whole cells, reported in the present paper, seem in fact to indicate that the primary lesion is not, or is not exclusively, at the lysosomal level: the inhibition of oxygen consumption caused by photosensitization after incorporation of neutral

red does not seem to be identifiable with the inhibition by heat, since the two phenomena are additive, nor is the onset of inhibition by heat shortened by previous exposure to light. The experiments with trypan blue, on the other hand, point out that, although this dye is able to maintain for some time the linearity of cellular respiration at 38°C, it cannot, at any of the concentrations tested, modify in any way the effect of exposure to 43°C. If the release of trypan blue from heat-damaged lysosomes were to contribute to the inhibition, at 43°C, of oxygen uptake, thus mimicking the effect of release of lysosomal enzymes, it would hardly be conceived that neither onset nor magnitude of this inhibition would vary with concentration of the dye.

It may be concluded that although the specific heat sensitivity of neoplastic cells is reflected, at least partially, at the level of their lysosomal membranes, this is hardly the primary mechanism of the cellular damage caused by the higher temperatures.

On the other hand, the greater lability of lysosomes from neoplastic cells may reflect just another aspect of the peculiar behaviour of the membranes of these cells to many agents. Previous results [3, 4] had shown that hepatoma cells are particularly sensitive to the action of filipin, a polyene antibiotic which increases the permeability of phospholipid-cholesterol membranes [17, 18]. Lysosomal and plasma membranes seem however to be both affected by this agent [19, 20], owing probably to similarities in their chemical composition [21]. Experiments with corticosteroids as stabilizers of lysosomal membranes would not be conclusive, due to their action also on plasma membranes [22].

Since preliminary studies on membrane function [23] have shown that the rate of transcellular migration of $^{14}$C-glutamate acid is heat-sensitive in hepatoma cells, but not in normal liver cells, it is probable that, in neoplastic cells, heat causes the labilization of both types of membranes, but that the specific damage due to the higher temperature is mainly or exclusively a consequence of alterations of the plasma membrane.

## SUMMARY

*Lysosomes from Novikoff hepatoma cells show, if compared to those from normal or regenerating liver cells, a higher lability, as measured by the rate of release of acid hydrolases. Moreover, in lysosomes from neoplastic cells, the release of the various enzymes at 38 and 43°C follows a non-homogeneous pattern. Neither intracellular lysosomal damage by photolysis, nor intralysosomal inhibition of acid hydrolases by trypan blue modify the onset of inhibition of respiration of tumor cells by elevated temperatures. It is concluded that the specific heat sensitivity of tumor cells is partially reflected at the level of their lysosomal membranes, but that is probably not the primary mechanism of the cellular damage caused by heat.*

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# Synergistic Effects of X-Rays and Drugs on a Human Tumor Xenograft, GW-39

DAVID M. GOLDENBERG and ERIKA AMMERSDÖRFER

*Departments of Pathology, University of Pittsburgh School of Medicine and Veterans Administration Hospital, Pittsburgh, Pennsylvania* 15240; *and Cancer Research Laboratory, Department of Medicine, University of Erlangen-Nuremberg, Erlangen, Fed. Rep. of Germany*

## INTRODUCTION

AT PRESENT, there is little convincing clinical evidence in support of chemotherapeutic potentiation of irradiation in the treatment of cancer. There have been some reports indicating synergistic effects for certain agents when combined with irradiation in specific neoplasms in man and in animals [cf. reviews 1–7]. However, few studies, either clinical or experimental, have considered whether the efficacy of both therapeutic modalities in combination might perhaps be more a function of the dosage of each rather than of the kind of agents used. It is for these reasons that we have undertaken an examination of the tumor growth-inhibitory effects of a number of anticancer agents possessing different mechanisms of action, and currently of clinical interest, in combination with X-radiation. It was also of interest to determine whether we could, by this means, improve the antitumor effects of certain drugs to which one of our recently established human tumor systems is relatively unresponsive.

## MATERIAL AND METHODS

The transplantable tumor used in this study is the GW-39 tumor established by us in 1966 from the surgical specimen of a patient's adenocarcinoma of the sigmoid colon, and since then grown continuously in the cheek pouch of unconditioned adult golden hamsters [8]. This tumor line has a 100% take incidence in the unconditioned hamster, regardless of transplantation site [9], and has not been propagated for more than 70 transplant generations. In terms of growth-behavior, GW-39 tumors are generally only locally expansive and attain large sizes at a fairly consistent and constant rate of growth. Their doubling time, based on plotting increase in tumor size with time during the first two weeks post transplantation, is about 3 days. Histologically, the tumor is a fairly well-differentiated carcinoma with round to ovoid cells containing cytoplasmic mucin of the signet-ring type (Fig. 1). The cells tend to grow in an alveolar or organoid pattern, being similar in appearance to the original donor's colonic carcinoma [8].

Twenty-day old cheek pouch tumors were excised and homogenized in isotonic saline containing penicillin (100 U/ml) and streptomycin (0·6 mg/ml) to an end dilution of 20% (w/v). One-tenth of a ml of this suspension was injected into both cheek pouches of male and female hamsters (*Mesocricetus auratus*) weighing 50–70 g. The animals were fed Altromin chow, chicken feed, and twice-weekly supplements of wheat germ and carrots; no drinking water was supplied.

After transplantation, the animals were randomized into groups of 10–15; one group served as controls, receiving the drug vehicle

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i.p. and manipulation of their cheek pouches as performed in those receiving X-rays, while the rest received various forms of therapy, either singly or combined. X-radiation was only applied to the left cheek pouch, while the opposite one was merely everted and retained exposed for the same period as required to irradiate the treated pouch. One half of each dose (usually 150 r) was applied from above and then, by rotating the radiation head 180°, from below the everted cheek pouch.

Radiation was delivered from a Siemans Dermopan unit 29 kV and 25 mA) at a rate in air of 841 R/min, with filtration of 0·3 mm Al; focus-tumor-distance was 15 cm. A cardboard was used to immobilize the cheek pouch for treatment. The rest of the animal was shielded with lead strips so as to minimize radiation scatter. X-radiation was always performed 5 days after the first tumor measurement, i.e. 10 days post transplantation or on the 3rd day of chemotherapy.

The following drugs were used in their commercial forms: Actinomycin C (Sanamycin), cyclophosphamide (Cytoxan), 5-fluorouracil (Fluoro-Uracile "Roche"), procarbazine hydrochloride (Natulan), and podophyllic acid ethyl hydrazide (SP-I, Proresid). Each agent was prepared fresh daily in physiological saline so that an amount of 1 ml per 100 g body weight was administered intraperitoneally once daily. Chemotherapy commenced on the 7th day after transplantation, or on the day of the second tumor measure (3 days prior to irradiation), and continued for a total of seven consecutive days with one injection daily. Toxicity in these experiments is expressed as a percentage of lethality of treated compared to control animals at 7 days post therapy. All combinations were tested in duplicate or triplicate in the 30–50th passages.

The growth-inhibitory effects of irradiation and/or chemotherapy were determined by comparing growth-rate (increase in tumor size against time) of treated and control tumors. Tumor size was obtained by measuring each tumor in three dimensions (length × width × depth) with a small caliper and using this product of the first measurement as the base value from which all successive products are expressed as simple multiples. These multiples of the tumor's original size were then averaged for each test group and recorded as mean growth-fractors. Growth-inhibition as a percentage is determined by the following formula:

$$GI = 100 - (GF_t/GF_c) \times 100,$$

where $GI$=growth-inhibition, $GF_t$=mean growth-factor of any treated group, and $GF_c$=mean growth-factor of the control tumors. The standard errors of these mean percentages of growth-inhibition have also been derived from the growth-factor data.

Measurements of tumor size were made on every second or third day during therapy, usually when the animals were anesthetized for other purposes, and again at 1, 4 and 7 days after therapy. Growth-inhibition has been determined at these latter times. Hexobarbital anesthesia was employed intraperitoneally.

Histological sections of both treated and control cheek pouch tumors were observed from time to time, so as to verify that increases in tumor size truly reflected increases in amount of well-preserved tumor tissue.

The combined effects of radiation and chemotherapy are considered as additive when the growth-inhibition range falls within that of the arithmetic sum of their individual percentages. No attempt is made here to differentiate between the terms *synergism, potentiation, augmentation* or *enhancement;* any significant improvement above the expected sum of each mode's individual inhibition range without likewise increasing host-toxicity is defined here as synergism for the two.

## RESULTS

The responsiveness of GW-39 to X-irradiation alone is summarized in Table 1 in terms of growth-inhibition percentages. Retardation of growth rate first appears after a single dose of 500 r, and improves, but not proportionally, by increasing the dose to 1500 r. Even at the maximum dose given, only a temporary inhibition of growth could be obtained for at least up to one week post therapy; indeed, no complete regressions could be documented in this series. Upon examination of the growth curves of the non-irradiated contralateral cheek pouches of animals in which one side received X-ray therapy (Table 1 and Fig. 4), no evidence for any systemic or indirect tumor-inhibitory effects could be seen.

The effects of a medium dose of radiation combined with a low dose of the ethyl hydrazide derivative of podophyllic acid, Proresid, are shown in Fig. 5. At best, only an additive effect can be interpreted for the combination group. This same result seems to be the case for a combination of a low dose of radiation with a relatively high dose of this drug (Fig. 6). Reducing both, however, to levels of each modality in which little tumor inhibition is achieved when given individually reveals that synergism is attained for the combination

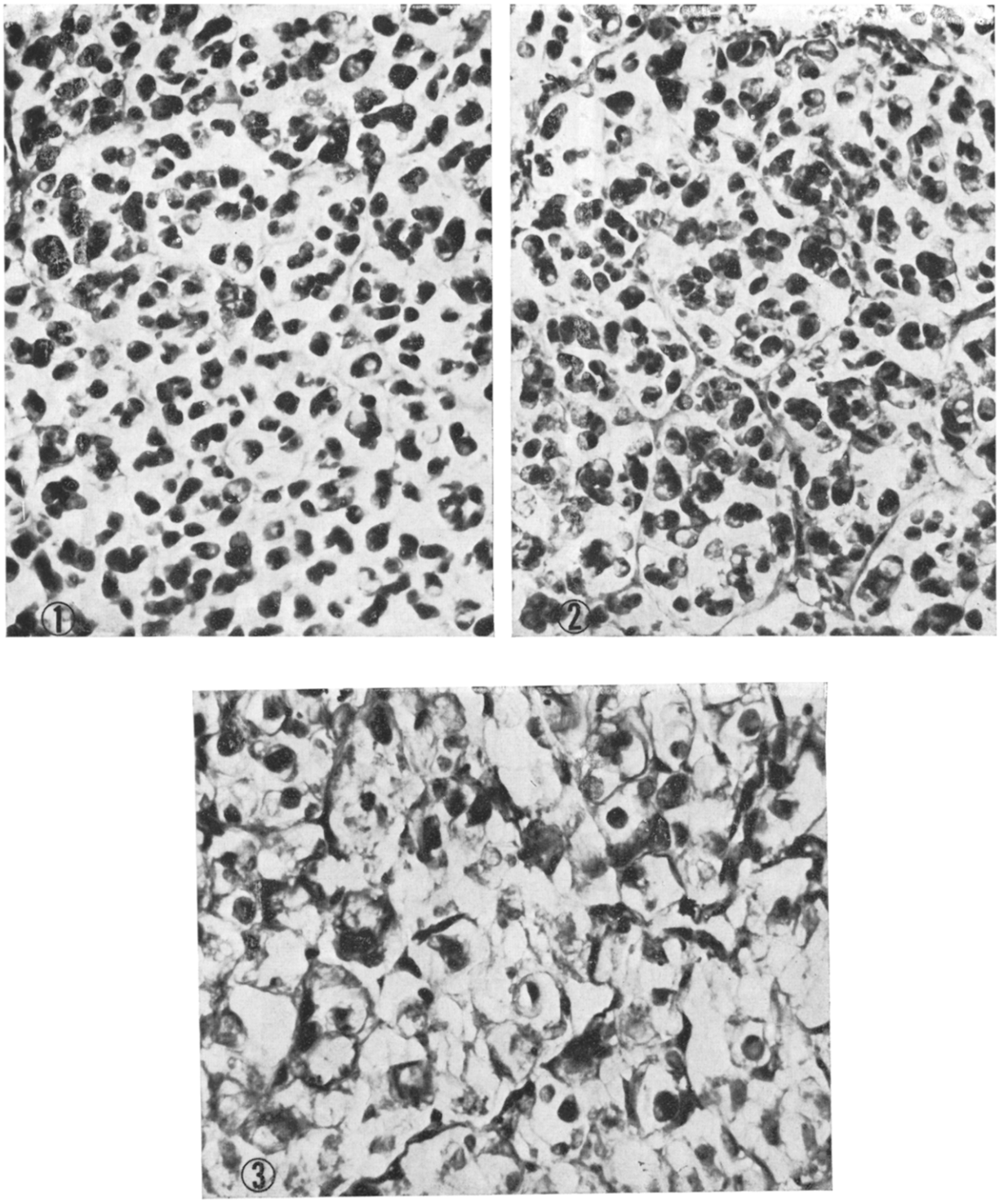

*Fig.* 1. *Section of a non-irradiated GW-39 human colonic carcinoma growing in a hamster cheek pouch for* 22 *days. H. & E.* × 120.

*Fig.* 2. *Photomicrograph of GW-39 cheek pouch tumor* 11 *days after irradiation with* 300 *r. H. & E.* × 120.

*Fig.* 3. *Microscopic morphology of a GW-39 cheek pouch tumor* 7 *days after combined X-irradiation* (300 *r*) *and actinomycin C therapy* (7 × 30 *mcg/kg*). *H. & E.* × 120.

(to face p. 74)

*Table* 1

| X-ray dose (R) | Percent growth-inhibition (Days after therapy) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | 5 | | 8 | |
| | Irrad. | Non-irrad. | Irrad. | Non-irrad. | Irrad. | Non-irrad. |
| 300 | 7 | 3 | 7 | 0 | 5 | 2 |
| 500 | 42 | 3 | 60 | 2 | 59 | 2 |
| 600 | 57 | 8 | 63 | 3 | 67 | 0 |
| 1000 | 36 | 6 | 54 | 5 | 69 | 0 |
| 1500 | 44 | 2 | 58 | 2 | 76 | 2 |

(Fig. 7), at negligible host-toxicity. Moreover, the level of growth-inhibition obtained for the combination compares well with that achieved with higher levels of X-radiation given alone (Table 1).

Because the radiopotentiating effects of the former agent could best be demonstrated at a low radiation dose of 300 r, this level was then preferred in the remaining experiments, although higher doses were tried for most of the combinations. As in the case of Proresid, 300 r of X-rays combined with low doses of fluorouracil (Fig. 8), cyclophosphamide (Fig. 9), actinomycin C (Fig. 10), and procarbazine hydrochloride (Fig. 11) resulted in synergistic antitumor effects. The most potent combination, without a corresponding increase in host-toxicity, was that of 300 r with 30 μg/kg body weight of actinomycin C (Fig. 10), yielding an 80% tumor growth-inhibitory level.

Histologically, the cheek pouch GW-39 tumors did not show any morphological changes following 300 r of X-radiation (Fig. 2), which is consonant with the lack of consistent growth-inhibitory effects of this dose (Table 1). When combining this amount of radiation with a low growth-inhibitory dose of actinomycin C (30 μg/kg body weight per day), degenerative changes including cytoplasmic vacuolization, disruption of cell membranes, nuclear fragmentation, and necrosis could be seen (Fig. 3).

## DISCUSSION

The serial propagation of a human colonic carcinoma in the cheek pouches of unconditioned adult golden hamsters provides a model for studying, under controlled conditions, the effects of various therapeutic measures on its growth rate and transplantability [9, 10]. Moreover, this model permits comparison of

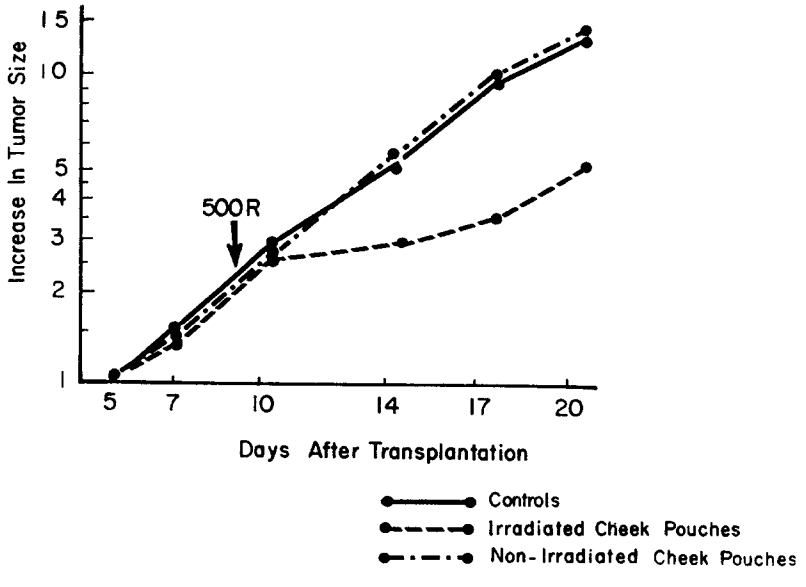


*Fig. 4. Mean growth curves for groups of irradiated, non-irradiated (contralateral), and control cheek pouch tumors.*

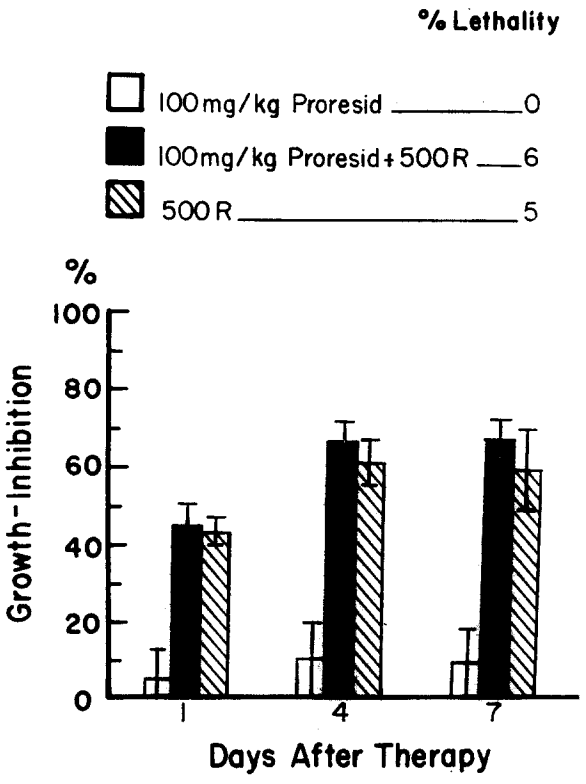


*Fig. 5. Growth-inhibition of GW-39 tumors at 1, 4 and 7 days post therapy with podophyllic acid ethyl hydrazide (Proresid), X-rays (500 r), and both combined. Vertical bars represent S.E. of the means.*

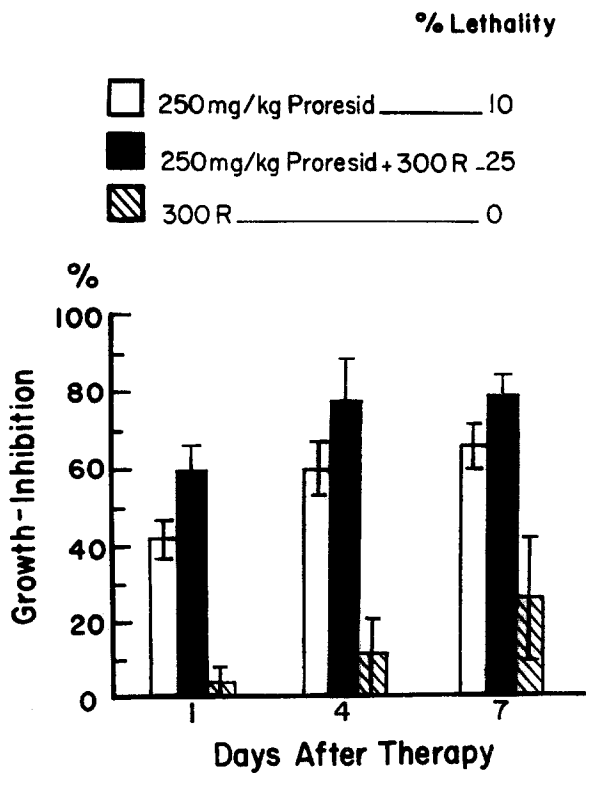


*Fig. 6. Growth-inhibitory effects of 250 mg/kg Proresid, 300 r X-rays, and both combined on GW-39 tumors.*

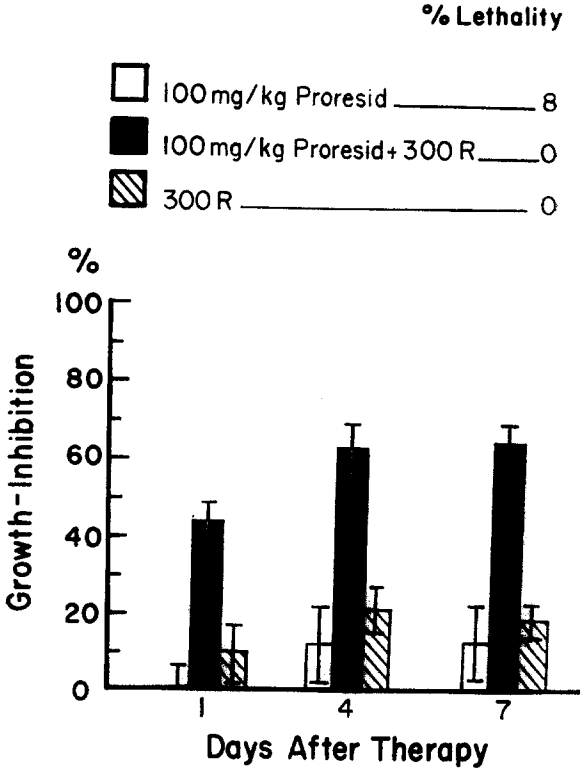


*Fig. 7. Synergistic growth-inhibitory effects of a combination of low doses of X-irradiation and Proresid.*

the effects of the combined treatment of one cheek pouch tumor with those of the remaining tumor in the other pouch of the same host receiving chemotherapy alone. Irradiation of one cheek pouch tumor in an animal bearing two tumors also provides a system for evaluating possible systemic or abscopal effects on the non-irradiated neoplasm.

The use of human tumors in the cheek pouch of the hamster for evaluating the effects of irradiation was first described by Warren and Gates [10], who claimed that human tumors growing in this site respond to irradiation as do their comparable cancer types in man. Likewise, the transplantable human colonic carcinoma used in these experiments was found to be as refractive to X-irradiation as this type of cancer in man is generally acknowledged to be, for only a temporary growth-retardation of about 58–76% could be achieved at 5–8 days post therapy respectively with radiation doses of up to 1500 r delivered directly to the everted cheek pouch tumor. Based upon cell viability of the irradiated tumor upon retransplantation it is interesting to note that Tovell [11], using a human epidermoid carcinoma in the hamster cheek pouch, also found that the dose necessary to achieve a 50% cell destruction rate to be 1500 r. In spite of such growth-inhibitory

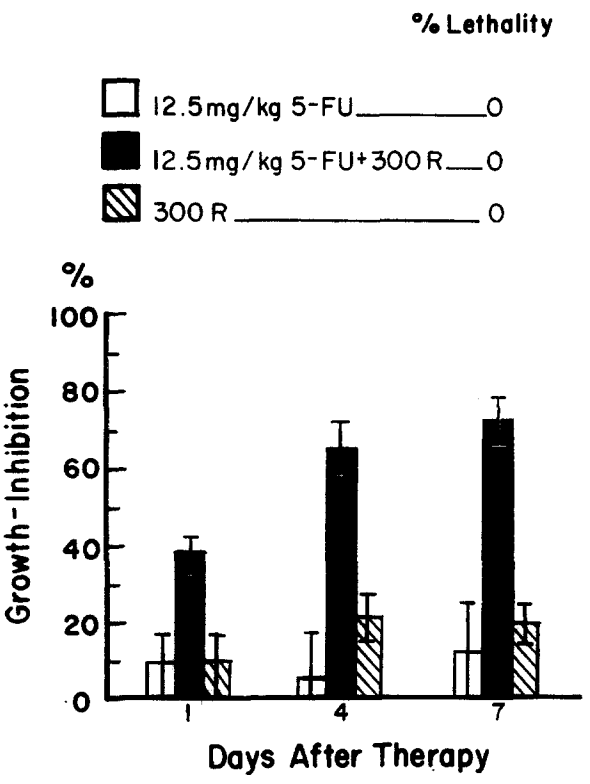


FIG. 8.

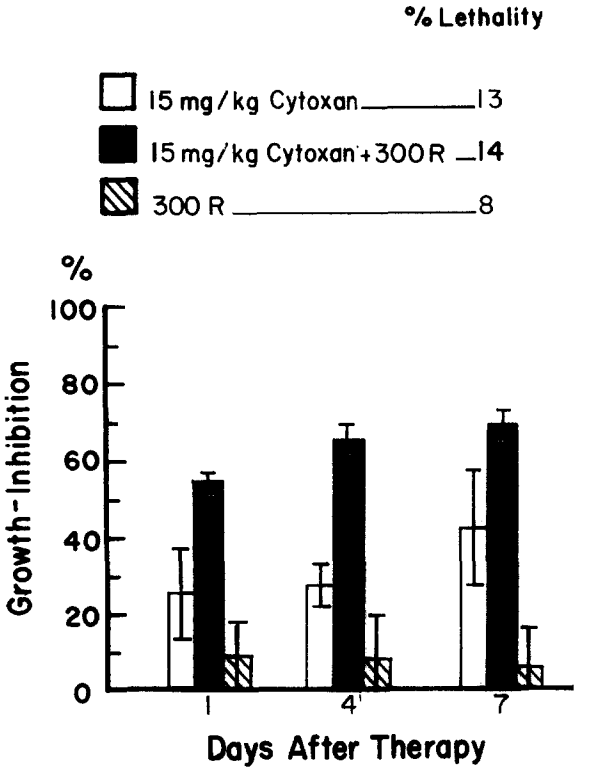


FIG. 9.

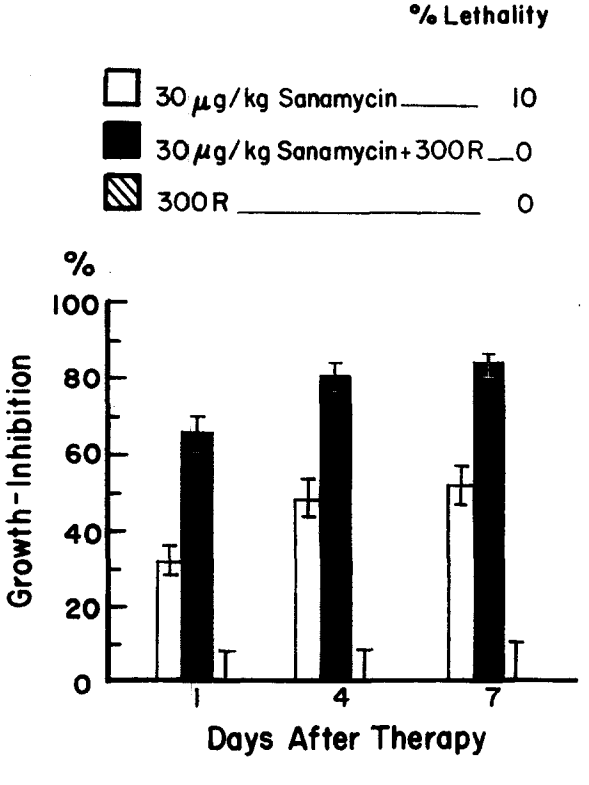


FIG. 10.

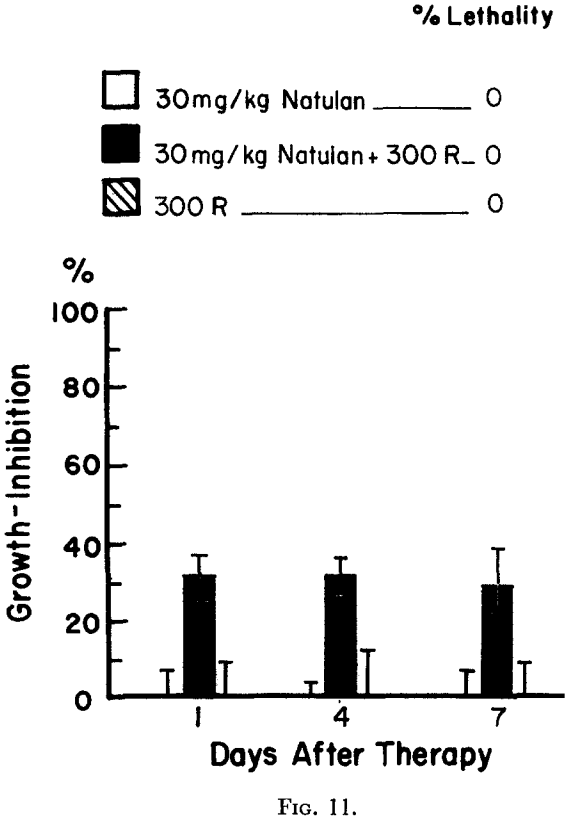


FIG. 11.

*Figs.* 8–11. *Synergistic growth-inhibitory effects of combinations of low doses of X-irradiation and chemotherapy with the following agents:* 5-*fluorouracil* (*Fig.* 8), *cyclophosphamide* (*Fig.* 9), *actinomycin C* (*Fig.* 10), *and procarbazine hydrochloride* (*Fig.* 11).

effects on the irradiated tumors, we did not observe any such alteration of growth rate of the opposite non-irradiated cheek pouch tumors, indicating that, at the doses employed, no indirect or systemic antitumor effects of X-irradiation are produced. This does not, of course, preclude the presence of other abscopal effects [4, 12].

Additional support for using human tumor xenografts for such therapeutic purposes can be found in the work of Kaufman and coworkers. Using xenografted human bladder tumors, Kaufman and Lichtenauer [13] recently reported experiments with 5-fluorouracil and irradiation in which similar synergistic effects as found in these studies were observed. Moreover, they claim that an enhancement effect of both modalities combined was witnessed clinically.

The crucial question in employing therapeutic combinations is whether the therapeutic ratio has been increased, for in many instances increased antitumor action of combination therapy is at the expense of greater damage to normal tissues and thus comparable to increasing the dose of either method alone. In examining the effects of combined regimens of X-rays and drug therapy, it was thus our concern to determine whether combinations of low levels of each parameter could yield comparable or better results than each alone at a higher therapeutic dose. The distinction between addition of effects and true synergism is clearly drawn in these experiments; the results with low drug dosages represent synergism because the total effect of drug and irradiation is greater than the sum of the two used independently. This principle has been found true for all of the five drugs tested, in spite of their being purported to possess different primary mechanisms of cytotoxic action. It would seem, then, that at proper dosage ratios, any cancerostatic agent can yield synergistic effects with irradiation, and that instead of speaking of "radiopotentiating" agents *per se*, the expression synergizing dose-ratios of such chemicals in combination with ionizing radiations would be more appropriate.

Why low doses of two cytotoxic modalities yield synergistic effects, while higher doses do not, is not clear. Perhaps combining low doses of two measures not only permits addition of cytotoxic effects within the tumor cell population, but also provides an opportunity for the summation of sublethal actions of each. Merely increasing their respective dosages does not necessarily guarantee that a proportionally higher cytotoxic effect is achieved for, within the limits of host-tolerance to therapy, we assume that only a specific percentage of cells responds to any given therapeutic measure.

In certain instances, such cancerostatic agents interfere with mechanisms of cell repair to sublethal doses of radiation. For example, recovery has been inhibited by administering actinomycin D [14] or methotrexate [15] to the irradiated cells, yet not by 5-fluorouracil [15]. It would appear, then, that such cytotoxic chemicals do not act uniformly on cell recovery mechanisms to radiation damage and, accordingly, that this is probably not a major factor contributing to the synergistic effects witnessed in this study.

Another possible explanation for the synergistic actions of low doses of X-rays and cancerostatic drugs is that irradiated neoplastic cells might lack some of the enzymes necessary for the catabolism of certain compounds, or *vice versa*. Under such circumstances, low therapeutic doses would exercise a somewhat selective antitumor effect while at the same time exerting minimal, if any, toxic effects to the host's non-irradiated normal tissues.

Finally, low doses of certain cytotoxic agents which are immuno-suppressive at higher levels might induce an enhanced immunological response to the tumor. It could be postulated that with lower cytotoxic doses of either modality, a small quantity of antigen released might stimulate immune mechanisms into action [16], whereas the sudden release of larger amounts of antigen, as may result from larger doses of X-irradiation and/or chemotherapy, could, if anything, even lead to immunological paralysis [17, 18]. Indeed, this paradoxical phenomenon might be relevant in the xenogeneic situation, especially since the host-response has been found to play an important role in the treatment of both isogeneic and allogeneic neoplasms [19–25]. However, neither rejection, either spontaneous or induced, nor total regression of this GW-39 tumor has as yet been witnessed in its hamster host, regardless of the therapeutic measures employed.

The pattern of response to low doses of the agent combined with X-rays has been found to be basically the same, yet the degrees of efficacy of the combinations do conform with the growth-inhibitory properties of these agents when given alone against the GW-39 tumor [26]. Further, the alkylating agent employed, cyclophosphamide, also proved to be synergistic with X-irradiation, thus supporting other evidence [27–30] that these agents are not truly radiomimetic in action.

We hesitate making analogies based on the evidence obtained in one such human tumor xenograft system, particularly where an immunological disparity between graft and host might come into play. Nevertheless, these results do suggest that even with the more chemo- or radioresistant solid tumors, such as colonic carcinomas, one should consider combining the drug(s) considered to be most effective in a particular tumor type with ionizing radiation. Contrary to the concept of maximum drug for maximum cell-kill, the best therapeutic ratio of a combination of modalities may conceivably be achieved with low doses of each, especially when possible concurrent host-defenses to the tumor might be present.

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## SUMMARY

*A human colonic carcinoma serially transplantable in cheek pouches of adult, unconditioned, golden hamsters, GW-39, has been found to be relatively unresponsive either to X-irradiation or drug therapy. Upon irradiation of one cheek pouch tumor in shielded animals, no systemic or abscopal growth-inhibitory effects on the opposite cheek pouch tumor were observed. Combinations of* 300 *r X-rays and either actinomycin C, cyclophosphamide, 5-fluorouracil, podophyllic acid ethyl hydrazide, or procarbazine hydrochloride yield comparable synergism of antitumor effects in this tumor system when low doses of each modality are given. It appears that the primary mechanisms of action of these different drugs is not of consequence in determining their possible synergism with X-irradiation. These results suggest that the most active drug in a particular kind of cancer be combined, at a low dosage level, with modest quantities of irradiation, even in neoplasms of a chemo- or radioresistant nature, such as colonic cancer.*

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# The Antitumoral Activity of Some Derivatives of 6-Aminochrysene*

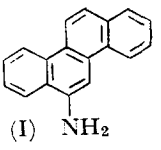
H. J. TAGNON†, ANDRÉ COUNE†, SYLVIO GARATTINI‡, R. ROSSO‡, G. LAMBELIN§, M. GAUTIER§ and N. P. BUU-HOÏ¶

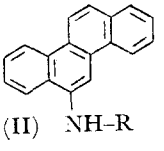
## INTRODUCTION

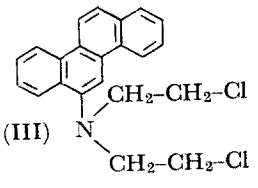
6-AMINOCHRYSENE (I) is known to inhibit the growth of solid tumors including spontaneous mammary adenocarcinoma [1, 2] and methylcholanthrene-induced carcinogenesis [1] in mice; in advanced human breast cancer, oral administration of this polycyclic amine has produced an objective regression in 2 cases out of 32 [3].

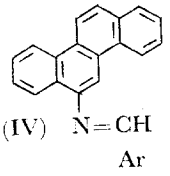
It is devoid of carcinogenic activity in mice and rats, on painting, subcutaneous injection, and ingestion [1, 4], although when injected in neonatal mice in their first three days of life it has produced tumors [5]. However, this is a rather aspecific testing method, non-carcinogenic compounds having given positive results [6, 7].

In both animal studies and in human patients, it has been shown that 6-aminochrysene is very poorly resorbed by the gut, a fact which might to some extent explain its greater efficacy in mice, to which it was administered intraperitoneally, than in humans [3]. These considerations led us to prepare and investigate the potential antitumor activity of a large number of derivatives of the amine (I), some of which show better solubility in water, and others in lipids, than the parent compound. The substances were tested in several types of tumors.




## PREPARATION OF COMPOUNDS

Twenty-one compounds, derived from 6-aminochrysene by substitution of the amino group, have been synthesized, and a twenty-second substance (GECA 371) was kindly supplied to us by Dr. W. Ross, from the Chester Beatty Cancer Research Institute, London. These substances are listed in Table 1.

(1) *N-(6-chrysenyl) succinamic acid* (m.p. 249–250°C) and *N-(6-chrysenyl)-α,β-diacetoxysuccinamic acid* (m.p. 152–153°C) were readily prepared by reacting 6-aminochrysene with succine anhydride and α,β-diacetoxysuccinic anhydride respectively. The solubility of the salt GECA 356 in water at 25°C was ≥0·5 g/ml (pH= 7–7·5). The ultraviolet absorption bands at 227, 261 and 270 mμ (log $\varepsilon_{270}$=5·061), and are very close to that of 6-aminochrysene itself, which showed absorption bands at 230, 243 and 275 mμ (log $\varepsilon_{275}$=4·684).

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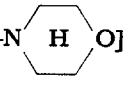
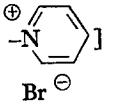
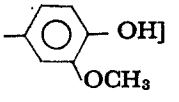
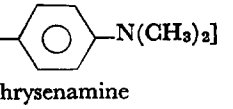
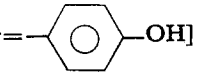
†Service de Médecine et d'Investigation Clinique, Institut Jules Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, Belgium.
‡Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62, Milano, Italy.
§Continental Pharma, S.A., Machelen. Bt, Belgium.
¶Institut du Radium, 26, rue d'Ulm, Paris, France.

*Table* 1.

| GECA No. | |
|---|---|
| 116 | 6-aminochrysene: [I] |
| 118 | N-(6-chrysenyl)-carbamic acid, ethyl ester [II; R=$-CO_2C_2H_5$] |
| 334 | 6-chrysenaminomethanesulfinic acid, sodium salt [II; R=$-CH_2-SO_2Na$] |
| 335 | N-(6-chrysenyl) succinamic acid [II; R=$-CH_2-CH_2-CO_2H$] |
| 356 | N-(6-chrysenyl)-α,β-diacetoxysuccinamic acid, sodium salt [II; R=$-CO-CH(OCOCH_3)-CH(OCOCH_3)-COONa$] |
| 357 | N-(6-chrysenyl) carbamic acid, hexadecyl ester [II; R=$-CO_2C_{16}H_{33}$] |
| 358 | 6-chrysenaminomethanesulfonic acid, sodium salt [II; R=$-CH_2-SO_3Na$] |
| 359 | 1-(6-chrysenamino)-3-phenylpropane-1,3-disulfonic acid, disodium salt [II; R=$-CH(SO_3Na)-CH_2-CH(SO_3Na)-C_6H_5$] |
| 499 | N-[bis(β-hydroxyethyl)aminoacetyl]-6-chrysenamine [II; R=$-CO-CH_2N(CH_2CH_2OH)_2$] |
| 500 | N-piperidinoacetyl-6-chrysenamine [II; R=$-CO-CH_2-$  ] |
| 501 | N-morpholinoacetyl-6-chrysenamine [II; R=$-CO-CH_2-$  ] |
| 502 | N-butylaminoacetyl-6-chrysenamine [II; R=$-CO-CH_2-NH-C_4H_9$] |
| 503 | 1-(6-chrysenylcarbamoylmethyl)pyridinium bromide [II; R=$-CO-CH_2-$  ] |
| 504 | N-diethylaminoacetyl-6-chrysenamine [II; R=$-CO-CH_2-N(C_2H_5)_2$] |
| 505 | N-diallylaminoacetyl-6-chrysenamine [II; R=$-CO-CH_2-N(CH_2-CH=CH_2)_2$] |
| 506 | N-octylaminoacetyl-6-chrysenamine [II; R=$-CO-CH_2NH-C_8H_{17}$] |
| 507 | N-(β-hydroxyethylamino)acetyl-6-chrysenamine [II; R=$-CO-CH_2-NH-CH_2-CH_2OH$] |
| 578 | N-(4-hydroxy-3-methoxybenzylidene)-6-chrysenamine [IV; Ar=  ] |
| 579 | N-(6-chrysenyl)oxamic acid, ethyl ester [II; R=$-CO-CO_2C_2H_5$] |
| 580 | N-(6-chrysenyl)malonamic acid, ethyl ester [II; R=$-CO-CH_2-CO_2C_2H_5$] |
| 581 | N-(4-dimethylaminobenzylidene)-6-chrysenamine [IV; Ar=  ] |
| 582 | N-(4-hydroxybenzylidene)-6-chrysenamine [IV; Ar=  ] |
| 371 | 6-[N,N-bis(β-chloroethyl)]aminochrysene [III] =(6-aminochrysene "mustard") |

(2) *Ethyl ester of N-(6-chrysenyl) carbamic acid* (m.p. 223–224°C) was prepared by the reaction of ethyl chloroformate on 6-aminochrysene in anhydrous pyridine; *Hexadecyl ester of the same acid* (m.p.129–130°C) was obtained from hexadecyl alcohol and benzyl carbamate by transesterification.

(3) *GECA* 335 was obtained from 6-aminochrysene and sodium formaldehyde-sulfoxylate; *GECA* 358 *and* 359 were similarly prepared with formaldehydrogen sodium sulfite and cinnamaldehyde-hydrogen sodium sulfite respectively.

(4) *GECA* 499 (m.p. 163–164°C), 500 (m.p. 172–173°C), 501 (m.p. 195–197°C), 502 (m.p. 160–162°C), 504 (m.p. 115–117°C), 505 (m.p. 122–123°C), 506 (m.p. 108–109°C) and 507 (m.p. 161–162°C) were prepared by condensation of 6-N-chloroacetamidochrysene with the appropriate amine; *GECA* 503 was obtained in a similar way from 6-N-bromoacetamidochrysene with pyridine.

(5) *GECA* 579 (m.p. 162–163°C) and *GECA* 580 (m.p. 200–202°C) were prepared by acylation of 6-aminochrysene with the appropriate acid ester.

(6) *The Schiff bases GECA* 581 (m.p. 194–197°C), 582 (m.p. 203–204°C) and 578 (m.p. 175–176°C) were prepared by condensation of 6-aminochrysene with *p*-dimethylaminobenzaldehyde, *p*-hydroxybenzaldehyde, and vanillin respectively.

Table 2 records the solubility characteristics of some of the compounds thus prepared, in comparison with those of 6-aminochrysene itself.

## TOXICITY TESTS

The $LD_{50}$ of the urethane GECA 118 and of the salt GECA 356 in mice (per os) was >8 g/kg. GECA 356 was however more toxic by subcutaneous (2·42 g/kg), intraperitoneal (0·445 g/kg) and intravenous injection (0·245 g/kg); in rats, the $LD_{50}$ of this compound was >4 g/kg by oral administration, and was 0·44 g/kg by intraperitoneal injection. In mice, and by oral administration, the $LD_{50}$ of GECA 357, 358 and 359 were ∽5 g/kg, ∽4·5 g/kg and >4 g/kg respectively. For GECA 359, the $LD_{50}$ by intraperitoneal, subcutaneous and intravenous injection was >1600 mg/kg.

## TESTS FOR ANTITUMORAL ACTIVITY

A. *Spontaneous mammary tumor in* ($C_3H/020)F_1$ *mice*

In addition to 6-aminochrysene itself, which was used as a reference substance, all the derivatives were tested on the spontaneous mammary tumor growing in ($C_3H/020$) $F_1$ mice.

The mice used were male castrated and treated with estrogens and female "force breed" purchased from the International Center for the provision and study of tumor-bearing animals, Netherlands. On arrival two diameters of the tumors were measured and tumor growth observed for a week; after this lapse of

*Table 2. Solubilities of 6-aminochrysene* and some of its derivatives*

| Compound | In water (25°C) | In olive oil (25°C) | In diethyleneglycol (25°C) |
|---|---|---|---|
| GECA No. | | | |
| 116 | <1 mcg/ml | ±1 mcg/ml | ±5 mcg/ml |
| 334 | 50 mcg/ml | — | — |
| 335 | 25–30 mcg/ml | — | — |
| 356 | ⩾500 mg/ml | — | — |
| 359 | ⩾300 mg/ml | — | — |
| 502 | — | — | 5 mg/ml |
| 506 | — | — | 5 mg/ml |
| 507 | — | — | 20 mg/ml |
| 504 | — | — | 15 mg/ml |
| 505 | — | — | 2·5 mg/ml |
| 500 | — | — | 5 mg/ml |
| 501 | — | — | 5 mg/ml |
| 357 | — | 800–900 mcg/ml | — |

*Determined by ultraviolet absorption spectroscopy.

*Table 3. Activity on spontaneous mammary tumors in* $(C_3H/020)F_1$ *mice*

| Compound No. GECA | Dose-i.p. (mg/kg) | mice No. | Body weight change in respect to controls (g) | Tumors per mouse *T/C* | Mortality (%) | Tumor weight inhibition *T/C* |
|---|---|---|---|---|---|---|
| 116 (6 amino-chrysene) | 100 | 36 | −2 | 0·79 | 11 | 0·35* |
| | 50 | 13 | −8 | 0·95 | 0 | 0·52† |
| | 25 | 13 | +4 | 0·84 | 8 | 0·68 |
| | 300‡ | 15 | −4 | 1·18 | 0 | 0·51† |
| | 100‡ | 15 | −3 | 0·91 | 0 | 0·60 |
| 118 | 500 | 12 | −2 | 0·60 | 40 | 0·27* |
| | 250 | 13 | −2 | 1·00 | 0 | 0·59† |
| | 500‡ | 11 | −4 | 0·90 | 0 | 0·71 |
| 334 | 500 | 12 | −5 | 0·86 | 0 | 0·17* |
| | 300 | 24 | −3 | 0·71 | 0 | 0·25* |
| 357 | 500 | 24 | +6 | 1·00 | 0 | 0·61† |
| 499 | 500 | 13 | −6 | 0·70 | 0 | 0·21* |
| | 300 | 12 | −1 | 0·82 | 0 | 0·56 |
| 500 | 500 | 36 | −3 | 1·00 | 0 | 0·42* |
| 501 | 300 | 12 | −3 | 0·86 | 16 | 0·49* |
| 502 | 300 | 12 | −11 | 0·78 | 0 | 0·21* |
| 504 | 500 | 14 | | | 100 | |
| | 300 | 14 | −28 | 0·81 | 21 | 0·19* |
| | 250 | 12 | −13 | 0·62 | 33 | 0·25* |
| | 200 | 25 | 0 | 0·81 | 16 | 0·45* |
| 505 | 300 | 27 | −4 | 0·72 | 0 | 0·48* |
| 506 | 300 | 14 | −12 | 1·24 | 71 | 0·19* |
| | 150 | 12 | +3 | 1·24 | 0 | 0·29* |
| | 75 | 12 | +2 | 1·24 | 8 | 1·00 |
| 507 | 300 | 12 | −3 | 0·77 | 0 | 0·48† |
| 578 | 300 | 12 | 0 | 0·58 | 25 | 0·35† |
| | 200 | 12 | −7 | 0·89 | 16 | 0·31* |
| | 100 | 12 | +2 | 0·74 | 0 | 0·61 |
| 579 | 300 | 12 | −5 | 0·65 | 8 | 0·50* |
| 581 | 300 | 24 | −1 | 0·72 | 0 | 0·37* |
| | 100 | 12 | +4 | 0·83 | 0 | 0·59 |
| 582 | 500 | 12 | −7 | 0·86 | 8 | 0·44† |
| | 300 | 12 | −6 | 0·76 | 0 | 0·25* |
| | 100 | 12 | 0 | 0·86 | 8 | 0·64 |
| 335 | 300 | 11 | +11 | 0·90 | 8 | 0·85 |
| 356 | 300 | 10 | +6 | 0·80 | 0 | 0·68 |
| 358 | 250 | 10 | +10 | 0·92 | 0 | 0·75 |
| 359 | 500 | 10 | 0 | 0·85 | 0 | 0·90 |
| 503 | 500 | 12 | — | — | 100 | — |
| | 100 | 13 | — | — | 100 | — |
| | 25 | 14 | +15 | 1·00 | 0 | 0·95 |
| 580 | 300 | 12 | +6 | 0·95 | 8 | 0·77 |
| 371 | 250 | 12 | — | — | 100 | — |
| | 20 | 25 | +7 | 0·85 | 0 | 0·84 |

*$p<0·01$; †$p<0·05$; $T$=treated mice; $C$=untreated mice; ‡given orally.

*Table* 4.

| Number of mice total | ♂ | ♀ | Mean diameter (mm±S.E.) of single tumor at day 1 | 8 | 15 | 22 | % body weight change at day 22 | Number of tumors per mouse at sacrifice | Average tumor weight (g±S.E.) at the 22nd day per mouse | per tumor |
|---|---|---|---|---|---|---|---|---|---|---|
| 12 | 8 | 4 | 13·5 ±0·6 | 13·8 ±1·1 | 15·5 ±1·4 | 16·3 ±1·4 | +10 | 1·4 | 3·50 ±0·51 | 2·47 ±0·50 |
| 15 | 10 | 5 | 13·5 ±0·5 | 14·7 ±0·7 | 17·0 ±1·0 | 19·3 ±1·7 | +12 | 1·6 | 6·98 ±1·27 | 4·25 ±0·76 |
| 11 | 6 | 5 | 14·0 ±1·0 | 15·1 ±1·2 | 17·1 ±1·0 | 17·2 ±1·2 | +12 | 1·5 | 4·48 ±0·81 | 2·98 ±0·55 |
| 12 | 8 | 4 | 12·6 ±0·5 | 14·0 ±1·2 | 15·8 ±1·1 | 18·7 ±1·3 | +11 | 1·2 | 4·29 ±0·54 | 3·43 ±0·52 |

time; those mice which did not show any change in tumor volume were excluded from the experiment. The others were distributed among the experimental groups so that the male and female ratio was the same in each group, and submitted to the following treatment schedule:

1, 2, 3, 4, 5, —, —, 8, —, 10, —, 12, —, —, 15, —, 17, —, 19 day.

On day 1, 8, 15 and 22, measurements of three diameters of tumors were made, and total body weight was checked. On day 22 the animals were sacrificed and the weight of their tumor was measured.

Table 3 reports the tumor weight inhibitions, the body weight change in respect to untreated mice, the number of tumors per mouse and the mortality on the 22nd day.

In order to give an indication of how the tumor growth is proceeding, four groups of controls are reported as examples in Table 4.

The following conclusions on the activity of these compounds may be drawn:

(1) apart from 6-aminochrysene itself, whose activity was confirmed anew, fifteen of the derivatives produced an inhibition exceeding 50% of the average tumor-weight per mouse;

(2) at least 5 compounds (*GECA* 334, 499, 502, 506 and 581) show a considerable activity and they deserve further studies.

(3) it is also interesting to note that 6-aminochrysene "mustard", while showing the

*Table* 5. *Activity on rhabdomyosarcoma transplanted S.C. in WAG/Rij rats*

| Compound No. GECA | Dose (mg/kg/i.p.) | Body weight change in respect to controls (g) | Mortality (%) | Tumor weight inhibition *T/C* |
|---|---|---|---|---|
| 116 (6-amino-chrysene) | 100 | −26 | 0 | 0·25* |
| 118 | 100 | −18 | 30 | 0·41 |
| 334 | 100 | −13 | 0 | 0·35* |
| 357 | 100 | −1 | 10 | 1·00 |
| 500 | 100 | −6 | 0 | 0·51† |
| 502 | 100 | −1 | 0 | 0·48* |
| 504 | 100 | −2 | 10 | 0·74 |
| 578 | 100 | −7 | 0 | 0·39* |
| 579 | 100 | +4 | 0 | 0·73† |
| 581 | 100 | +2 | 0 | 0·48* |

*$p<0·01$; †$p<0·05$, $T$=treated rats; $C$=untreated rats.

Each group was formed by 10 rats. All compounds were suspended in peanut oil. The average weight of tumors at the sacrifice in the untreated rats ranged from 11·9±1·9 g to 19·7±1·4 g.

high degree of toxicity expected, is devoid of antitumor activity in this test.

B. *Rhabdomyosarcoma in inbred WAG/Rij rats*

6-Aminochrysene and several of its derivatives were studied on a syngeneic, slow-growing tumor generously supplied, together with the inbred rats, by Dr. D. van Bekkum (Radiobiological Institue TNO, Netherlands). A treatment schedule like that described above for the spontaneous mammary tumor has been followed; treatment started 30 days after the s.c. transplantation of the tumor, when the tumor diameter was about 15-20 mm.

Results summarized in Table 5 show that 6-aminochrysene and several of its derivatives, at the dose of 100 mg/kg i.p., were able significantly to affect the tumor growth. Compound 118 which is active, showed a strong toxicity (30% mortality). The activity of 6-aminochrysene and several of its derivatives in this test is the more noteworthy as this type of tumor has so far been found highly insensitive to chemotherapy.

C. *Sarcoma* 180 *and Ehrlich carcinoma in Swiss mice*

Swiss, random-bred mice, transplanted subcutaneously with 5 millions cells of other Sarcoma 180 or Ehrlich carcinoma, were treated starting 5 days after tumor implant, when tumor diameter was about 5 mm, and performed every other day during 2 weeks, seven injections being given on the whole.

None of the compounds tested according to this schedule showed an inhibitory effect on either tumor (see Table 6).

*Table 6. Activity on Ehrlich carcinoma and Sarcoma 180 implanted S.C. in Swiss mice*

| Compound No. GECA | Dose (mg/kg i.p.) | Tumor weight inhibitions $T/C$ | |
|---|---|---|---|
| | | Sarcoma 180 | Ehrlich carcinoma |
| 116 (6-amino-chrysene) | 100 | 1·18 | 0·98 |
| 118 | 500 | 0·68 | 0·95 |
| 334 | 300 | 1·15 | 0·86 |
| 500 | 500 | 1·22 | 0·94 |
| 501 | 300 | 1·35 | 0·99 |
| 504 | 300 | 1·15 | 1·13 |
| 505 | 300 | 1·11 | 1·05 |
| 506 | 150 | 1·12 | 1·19 |

$T$=treated mice; $C$=untreated mice.

Each group was formed by 10 mice.
The average weight of tumors in the untreated mice ranged from $0·63 \pm 0·09$ g to $0·91 \pm 0·14$ g for Sarcoma 180 and from $0·58 \pm 0·05$ g to $0·80 \pm 0·09$ g from Ehrlich carcinoma.

## DISCUSSION

The observations reported here on 6-aminochrysene and some of its derivatives are interesting because they introduce an entirely new line of chemical compounds with potential antitumor activity in humans. These compounds are characterized by a complete lack of bone marrow toxicity in the dosage used and by a somewhat specific action on two experimental tumors in animals: a rhabdomyosarcoma and a mammary tumor. Other tumors commonly employed in screening chemotherapeutic agents were not sensitive to the 6-aminochrysene family of compounds.

The only experience in men is the observation of 2 cases of objective regression in advanced mammary cancer [3]. More extensive study in other forms of tumors is now being pursued. One difficulty in the testing of these compounds is the marked gastro-intestinal intolerance and the poor absorption in humans. In view of the favorable effects of 6-aminochrysene and its derivatives in animals, a definite search for better tolerated compounds should be instituted.

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## SUMMARY

*Twenty-two* N-*substituted derivatives of the carcinostatic* 6-*aminochrysene have been tested as potential carcinostatic agents (using* 6-*aminochrysene as a standard) on spontaneous mammary tumor in* [$C_3H$/020] $F_1$ *mice, and on the slow-growing transplanted rhabdomyosarcoma in inbred WAG/Rij rats. Many of these compounds displayed important activity against both types of tumor, and some of them merit further investigations. In contrast compounds active on these experimental tumors, were completely inactive on Ehrlich carcinoma and on Sarcoma* 180.

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# Experimental Radiotherapy of a Rat Rhabdomyosarcoma with 15 MeV Neutrons and 300 kV X-Rays

## II. Effects of Fractionated Treatments, Applied Five Times a Week for Several Weeks

G. W. BARENDSEN and J. J. BROERSE
*Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands*

### INTRODUCTION

IN THE first paper of this series, studies have been reported of effects produced by single doses of 300 kV X-rays and 15 MeV neutrons in transplantable rhabdomyosarcomas, growing in the flanks of rats [1]. The responses which were investigated included variations of the fractions of cells capable of unlimited proliferation, tumour volume changes and proportions of tumours cured. In separate experiments, cell proliferation parameters, e.g. cell cycle times, growth fractions and cell loss factors, have been derived after irradiation, using autoradiographic techniques [2]. The results of these investigations have demonstrated that studies of a number of different parameters in a single type of experimental tumour provide insights in the responses at the cellular level which cannot be obtained from measurements of a single end-point only. Therefore the same methods were applied in studies of effects of fractionated irradiations, and the results are reported in the present paper.

In the design of experiments with fractionated treatments, a choice must be made with respect to possible combinations of fraction sizes, total doses and intervals between fractions. We have decided to start with investigations of effects of equal doses, administered five times per week for periods of several weeks. The results to be reported demonstrate that the comparison of effects produced by low-LET radiations, e.g. X-rays, employed in clinical radiotherapy, with effects produced by 15 MeV neutrons, is not only of interest with respect to the difference in the oxygen effect, but that the shapes of survival curves and corresponding differences in the repair of sublethal damage induced by these radiations are also important. It will be shown that the data obtained with fractionated treatments provide new insights in the responses of tumours to both types of radiation. In addition to these fundamental aspects, the treatment schedules employed are of practical importance because they are similar to those commonly employed in clinical radiotherapy.

In studies of fractionated doses of low-LET radiations, e.g. X-rays and high-LET radiations, e.g. fast neutrons, at least nine factors must be considered which may influence their relative biological effectiveness. These factors are:

(1) *Cell reproductive death as a function of the dose*

Each dose of radiation administered in a fractionated treatment renders a fraction of the

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cells incapable of unlimited proliferation. Because of the differences between the shapes of survival curves of cells irradiated with fast neutrons and X-rays, the RBE of fast neutrons relative to X-rays increases with decreasing dose fractions [1].

(2) *Repair of sub-lethal damage*

Cells which after a given dose of radiation have retained the capacity for unlimited proliferation sustain sub-lethal damage which renders them more susceptible to subsequent irradiation than unirradiated cells [3, 4]. Many types of cells are known to recover from this sub-lethal damage within a few hours after irradiation [3]. With fast neutrons this effect is reduced relative to X-rays and consequently the RBE depends on the sizes and numbers of fractions and on the time intervals employed [5–9].

(3) *Variation of radiosensitivity as a function of cell age*

This variation has been demonstrated in studies with synchronized cell cultures for different types of cells [10–13]. As a consequence cells surviving in a non-synchronous population irradiated with a given dose, may be partially synchronized. If a subsequent dose fraction is administered after an interval of e.g. 24 hr, the distribution of cell ages may not yet be randomized and this might influence the effectiveness of this dose. Since differences in sensitivity related to cell age are smaller for high-LET radiations as compared with X-rays, the RBE of 15 MeV neutrons may depend on the time sequence of post-irradiation progression of cells through the cell cycle and on the time intervals between dose fractions [14].

(4) *Mitotic delay*

Irradiated cells present a division delay which manifests itself as a block in the $G_2$ stage [15]. This delay is dependent on the radiation dose and on cell age. As a consequence irradiated cells may become synchronized. The RBE of fast neutrons for mitotic delay has not yet been investigated. Data obtained for $\alpha$-radiation and X-rays indicate that differences may be found between the RBE with respect to mitotic delay and the RBE for cell reproductive death. Such differences might influence the synchronizing effect of a given radiation dose. As noted earlier, this may cause differences in the responses of cells to subsequent doses [16].

(5) *Selective killing of cells*

Not all cells in a tumour may be expected to exhibit exactly the same radiosensitivity, i.e. dose-survival relationships may show a distribution of the values of extrapolation numbers $N$ or the mean lethal doses $D_0$. A fractionated treatment with small daily doses tends to damage selectively cells with a relatively small capacity for repair of sub-lethal damage. This selection, if present, will be less important after irradiation with fast neutrons as compared with X-rays, because of the smaller amount of sub-lethal damage induced by fast neutrons [7]. It is generally observed that differences in radiosensitivity between various cell populations, due to either external conditions or to intrinsic factors, tend to be smaller for 15 MeV neutrons as compared with X-rays [7].

(6) *Fractions of anoxic cells*

Investigations of a number of experimental tumours have shown that a fraction of the cells may be depleted in oxygen, rendering them more resistant to X-rays and gamma-rays by a factor of about 2·5 to 3·5 as compared to well-oxygenated cells [1, 17–26]. This dependence of radiosensitivity on the oxygen concentration is smaller for fast neutrons than for X-rays [27]. As a consequence of this difference, the RBE of fast neutrons for effects on tumours depends on the fraction of anoxic cells as well as on the dose of radiation employed [1, 6, 7, 8].

(7) *Reoxygenation of anoxic cells in irradiated tumours*

It has been shown that in a number of experimental tumours the oxygenation status of cells may change during a course of fractionated irradiation [24, 26, 28–32]. It is evident that the time course and extent of these changes and the magnitude of the daily dose fractions used, may influence the effectiveness of fractionated treatments with X-rays and fast neutrons differently.

(8) *Repopulation of the tumour by surviving cells*

Investigations of an experimental tumour have shown that cells which after a given dose of radiation have retained the capacity for unlimited proliferation, can start to multiply with a shorter cell cycle time than cells in the unirradiated tumour [2]. As a consequence of the increased proliferation rate, the recurrent growth of an irradiated tumour is found to be more rapid than expected on the basis of the growth parameters of the tumour before irradiation [1, 2, 33, 34]. This increase in the rate of cell production may depend on the extent and nature of the damage produced in the tumours and might conceivably cause

differences between effects produced by fast neutrons and X-rays.

(9) *Non-lethal damage*

Cells cultured *in vitro*, which after irradiation have retained the capacity for unlimited proliferation, produce clones with a wider distribution of numbers of cells per clone than unirradiated cells. In particular after large doses of radiation the number of cells per clone increases relatively slowly due to death of part of the progeny [35–37]. This phenomenon, denoted "non-lethal damage", may cause irradiated tumours to regrow slower than would be expected on the basis of the rate of cell production [1]. Experiments carried out with cultured cells have shown that the RBE of high-LET radiations for the induction of non-lethal damage after single doses is not significantly different from the RBE for induction of cell reproductive death. Whether this is true for fractionated irradiations has not yet been investigated in detail.

It will be clear from this brief discussion of factors which may influence the effectiveness of treatments of tumours with fractionated doses of different radiations, that the investigation of only a single parameter, e.g. the growth delay or the proportion of tumours cured or the fractions of surviving cells, will not provide enough data for the interpretation of differences between cellular effects produced by fast neutrons and X-rays, which is necessary to evaluate the possible advantages of fast neutrons in clinical radiotherapy.

In the studies reported in this paper, different responses of a rhabdomyosarcoma transplantable in an inbred strain of rats, treated with daily doses of 300 kV X-rays or 15 MeV neutrons, have been investigated. In subsequent papers responses produced by other fractionation schedules and by irradiation at low dose rates will be reported.

## MATERIAL AND METHODS

(a) *Biological techniques*

The origin and growth characteristics of the rhabdomyosarcomas transplantable in the inbred strain of WAG/Rij rats have been described previously [1, 2]. This tumour, denoted R-1, obtained by a special selection procedure, consists of cells which after excision of the tumour and application of a cell dispersion technique, can be cultured directly *in vitro*. Cells derived from unirradiated tumours give rise to clones with a plating efficiency of $35 \pm 10\%$ [1]. As described elsewhere in detail, this technique provides the possibility to assay changes in the fractions of clonogenic cells in tumours during and after a fractionated treatment [1].

The methods employed to measure tumour growth delay and proportions of tumours cured, have been described previously [1]. In the studies involving fractionated irradiations with multiple daily doses, measurements of tumour volumes were carried out three times per week from one week before the start of the experiment until after treatment the volume was equal to at least twice the volume present at the day of first treatment (day 0). Tumours which had regressed and could not be palpated at six months after the beginning of the treatment were considered to have been cured [1].

(b) *Irradiation techniques and dosimetry*

The apparatus used for the production of 300 kV X-rays and 15 MeV neutrons and the methods of dosimetry based on tissue equivalent ionization chambers have been described in the first part of this series [1]. The yield of $2 \times 10^{10}$ neutrons/sec of the neutron generator, obtained through the D–T reaction, has necessitated a limitation of the experiments to relatively low doses. As a consequence no cures could be obtained. A new generator is presently being installed which in the near future will provide the possibility to obtain higher dose rates and to employ thicker shielding for the protection of the animals [1].

## EXPERIMENTAL RESULTS

(a) *Changes of tumour volumes and numbers of clonogenic cells during and after three weeks of fractionated treatments*

In this series of experiments groups of animals were inoculated in one flank with small pieces of tumour. When these tumours had grown to a volume of between 0·5 and 1·0 $cm^3$, treatments with equal daily doses, five days per week, were started on a Monday and continued for three weeks. Daily doses of 200 rads or 300 rads of 300 kV X-rays and of 50, 70 or 100 rads of 15 MeV neutrons were applied. Tumour dimensions were measured three times per week. At different time intervals, during and after the treatment, two animals were sacrificed and their tumours were excised. From each tumour a cell suspension was prepared and the fraction of clonogenic cells was measured by the plating technique [1]. At least 5 and usually 10 animals were not sacrificed but kept alive until their tumours had grown to a volume equal to at least twice the volume at the start of the treatment.

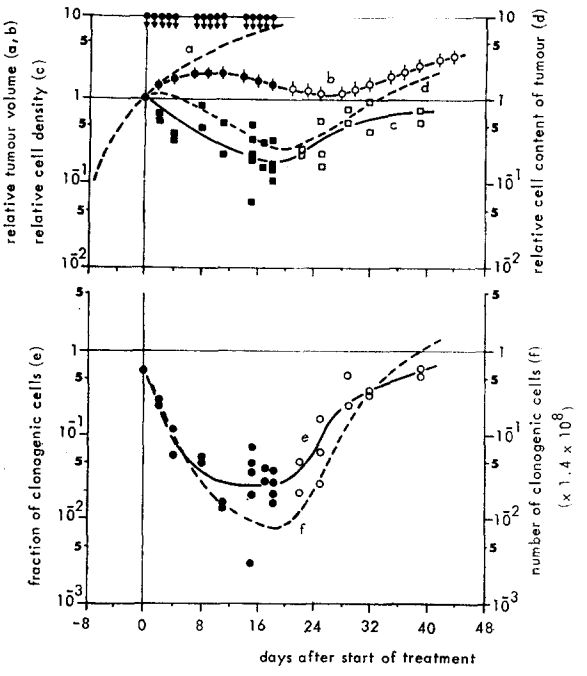


*Fig.* 1.

*Changes of tumour volume, numbers of cells and numbers of clonogenic cells in experimental R-1 rhabdomyosarcomas treated five times a week for three weeks with* 200 *rads of* 300 *kV X-rays.*

*Closed symbols refer to data obtained during treatment, open symbols to data obtained after treatment. Mean volume at start of treatment* $0 \cdot 8$ *cm*$^3$, *mean cell content* $1 \cdot 4 \times 10^8$ *cells.*

*Curve a: Growth curve of controls. Volumes relative to volume at start of treatment.*

*Curve b: Growth curve of irradiated tumours. Irradiations are indicated by arrows. Volumes relative to volume at start of treatment.*

*Curve c: Number of intact cells per gram of tumour, relative to value for unirradiated tumour of the same volume.*

*Curve d: Numbers of intact cells present in tumour, relative to numbers at start of treatment* ($4 \times 10^8$ *cells*).

*Curve e: Fraction of clonogenic cells in suspension prepared from irradiated tumour, relative to value for unirradiated tumour of the same volume.*

*Curve f: Numbers of clonogenic cells present in tumour at time of assay.*

In Fig. 1 data are presented for tumours treated with daily doses of 200 rads of 300 kV X-rays, administered five times per week for three weeks. Curve a represents the growth curve of unirradiated tumours and curve b shows the variation of the mean volume of the irradiated tumours during and after treatment. The vertical lines represent standard errors computed from the variation between values derived from at least 5 tumours [1]. The irradiated tumours continue to increase in volume during the first week of treatment and reach a mean volume equal to about twice the volume at the start of the treatment. During the second week of the treatment the volume remains constant and during the third week a decrease is observed. This decrease of the mean volume continues during the fourth week, i.e. during the first week after the end of the treatment. At the end of the fourth week the mean volume reaches a value approximately equal to that at the start of the irradiation. During the fifth week the tumours start to grow again and at day 37 the mean volume is equal to twice the volume at day 0. Since the volume of the unirradiated controls increases by a factor of 2 in 4 to 5 days, the tumour growth delay produced by the three weeks of treatment with 200 rads of X-rays per day is equal to about 33 days.

Curve e of Fig. 1 shows the variation of the fractions of clonogenic cells in the tumours as a function of the time after the start of treatment. The values have been computed relative to the plating efficiency of cells from unirradiated tumours, which served as a base line value [1]. This implies that an increase of the fraction of clonogenic cells to the value $1 \cdot 0$ corresponds to a return of this parameter to a value equal to that of unirradiated tumours. Although the cells from a suspension prepared from an excised tumour are always plated on 5 dishes, the standard errors which can be calculated from the variation in the numbers of clones per dish are not presented in the figure, because they usually amount to less than 10% and are much smaller than differences measured between individual tumours treated identically. These latter differences are mainly due to two factors, namely (a) experimental variations in the cell dispersion- and plating technique and (b) true differences in the properties of the tumours and their reactions to the radiation treatments. Differences between tumours may be caused by variations in fractions of anoxic cells and in growth fractions and are presumably related to the supply of blood and nutrients to the tumours. The relative importance of these factors cannot be derived directly from our data but the latter cause is certainly not negligible.

Curve e of Fig. 1 starts at day 0 at the value of $0 \cdot 61$, equal to the surviving fraction after a single dose of 200 rads of X-rays. During the first week of the treatment the fraction of clonogenic cells decreases rapidly, but during the second week the decrease is smaller and during the third week the fraction remains approximately constant. After the end of the treatment the fraction of clonogenic cells increases rapidly for about two weeks and reaches a value equal to about 30% of that in an untreated tumour. Subsequently the value of the controls is approached more slowly and it is not known whether this value is reached completely.

The fractions of clonogenic cells, measured for the cell suspensions prepared from individual tumours, do not provide a direct measure of the total numbers of clonogenic cells in the tumours. In order to estimate changes in the total numbers of clonogenic cells in the tumours during and after the treatment, it is necessary to take into account changes of the cell densities and of the tumour volumes. After irradiations with single doses of 1000 rads and 2000 rads of X-rays and 600 rads of 15 MeV neutrons, the yields of the cell dispersion technique could be used as a measure of the cell density in a tumour [1]. After these single doses the cell yields of the dispersion technique were decreased by only a factor of about 0·5 during a time interval after irradiation which depended on the dose, and subsequently an increase of the yield to the control value was observed. These data were found to be in good agreement with cell densities deduced from counting numbers of intact cells in histological sections. In the present experiments with fractionated doses, the cell yields obtained with the dispersion technique varied to a much larger extent than after single doses. The decrease of the yield during the treatment depends on the daily dose and the treatment period. Examination of histological sections has shown that although in these tumours the cell density is about 0·5 times the density in unirradiated tumours, the majority of the cells appears severely damaged. It is not possible to estimate from these sections the fractions of intact cells quantitatively.

The observation that the cell yield decreases to a considerably larger extent than the cell density observed in histological sections can be explained by the assumption that during the treatment with trypsin which is part of the dispersion procedure, many severely damaged cells are lysed. These cells are of little interest in the present studies since it is unlikely that they would be capable of unlimited proliferation. The cell yield of the dispersion technique applied to treated tumours relative to the yield for untreated tumours has been considered therefore as a measure of the number of intact cells present per gram of tumour, i.e. as a measure of the density of cells which are sufficiently intact to withstand the dispersion technique. The yield values are represented by the squares in the upper part of Fig. 1. As noted in the discussion of Part I of this series, the yield of the dispersion technique may vary for tumours treated identically and this is also shown by the scatter of the squares in Fig. 1. However, the results are sufficiently consistent to derive curve c for the mean variation of the cell density as a function of the time during and after the three weeks of treatment. Curve c shows that the mean value of the cell density decreases by a factor of about 6 during the three weeks of treatment with 200 rads of X-rays per day. After the end of the irradiation a slow increase is observed to a value which at three weeks after the end of the treatment is approximately equal to 0·7 times that of unirradiated tumours.

From the measured tumour volumes and relative cell densities presented in curves b and c respectively, it is possible to calculate the variation of the numbers of intact cells present in the tumours as a function of the time interval after the start of the treatment. This relation is represented by curve d of Fig. 1. Subsequently curve d can be used to calculate the numbers of clonogenic cells present in the tumours as a function of the time interval after the start of the treatment, given by curve f. The shape of curve f was thus derived by multiplication of the numbers of intact cells present in the tumours (curve d) and the fractions of clonogenic cells assayed by the cloning technique (curve e). Tumours with a mean volume of 0·8 $cm^3$ contain an average number of $1·4 \times 10^8$ clonogenic cells [1]. Curve f shows that this number decreases by approximately a factor of 10 during the first week of the treatment, while during the second week this factor is about 6 and during the third week about 2. These results indicate that the effectiveness of the irradiation with 200 rads of X-rays per day for 5 days per week diminishes during the three weeks of the treatment. It can further be deduced from the shape of curve f that after the end of the three weeks of treatment, the numbers of clonogenic cells start to increase very rapidly during a period of about two weeks, corresponding to a mean doubling time of approximately 2·7 days. The rate of increase is smaller during the third week, corresponding to a doubling time of about four days. Numerical values have been summarized in Table 1.

In Fig. 2, results are presented for tumours treated five times per week for three weeks with daily doses of 300 rads of 300 kV X-rays. Curves a and b represent the mean volume changes of untreated and treated tumours respectively. For treated tumours growth continues during the first week to a volume equal to about 1·8 times the volume at day 0. Subsequently a decrease in tumour volume is observed during the last two weeks of irradiation and for 10 days post-irradiation. The minimum volume reached is equal to 0·25 times the volume present at the start of the

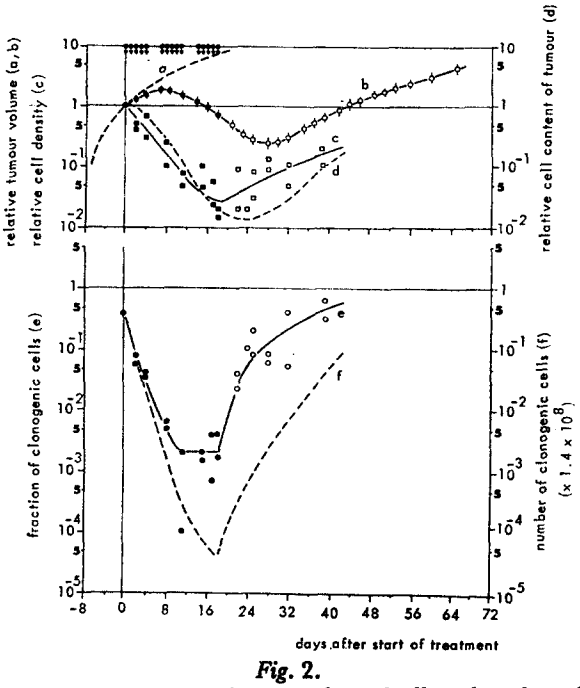


*Fig. 2.*

*Changes of tumour volume, numbers of cells and numbers of clonogenic cells in experimental R-1 rhabdomyosarcomas treated five times a week for three weeks with 300 rads of 300 kV X-rays.*

*For description of curves a to f and symbols compare Fig. 1.*

treatment. At the end of the second week post-irradiation the tumours resume growth again and at day 44 the mean volume has reached a value which is equal to that measured at the start of the treatment. It reaches twice that value at day 55, i.e. the mean volume doubling time of these recurrent tumours, is about 11 days as compared with 4 to 5 days for untreated tumours of the same volume. The growth delay derived as the difference in the time intervals required by treated and untreated tumours to reach twice the volume measured at the start of the experiment, is equal to 55—4=51 days.

Curve c of Fig. 2 represents the variation of the density of intact cells in the tumours derived from the yields of the cell dispersion technique. Curve d has been derived by multiplication of corresponding values of curves b and c as described earlier. Thus curve d represents the variation of the mean numbers of intact cells in the tumours relative to the number present at the start of the irradiation. In contrast to the case of 200 rads of 300 kV X-rays per day, the number of intact cells continues to decrease for a few days after the end of the treatment, indicating that cell loss exceeds cell production during this period. It is further of interest to note that the cell density and cell number start to increase earlier than the tumour volume.

Curve e of Fig. 2 represents the variation of the fraction of clonogenic cells in the tumours, measured by the cell dispersion- and plating technique. This fraction decreases during the first two weeks of treatment to a value of 0·002 and remains constant during the third week. Immediately after the end of the treatment a rapid increase is observed.

Curve f represents the variation of the number of clonogenic cells in the tumour, which has been derived by multiplication of corresponding values of curve d and curve e. This number decreases during the three weeks of treatment but starts to increase immediately after the last dose at a rate corresponding to a doubling time of about 1·9 day. Other numerical values are summarized in Table 1.

In Fig. 3 results presented are obtained by irradiating tumours 5 times per week for 3 weeks with daily doses of 50 rads of 15 MeV neutrons. The curves a, b, c, d, e and f represent the same parameters as described with respect to Fig. 1 and 2. Curve b shows that, during treatment with 50 rads of 15 MeV neutrons, the tumours continue to grow during the first two weeks. During the third week the volume remains approximately constant but, after completion of the three weeks of treatment, growth is resumed within a week. Curve d shows that the number of intact cells decreases by about a factor of only 2. It can be concluded from curve f that the number of clonogenic cells has decreased to about 3% of the pre-irradiation value by the end of the third week and subsequently increases with a doubling time of about 2 days. It is clear that the treatment with daily doses of 50 rads of 15 MeV neutrons is less effective than treatment with 200 rads of 300 kV X-rays per day.

In Fig. 4 results are presented derived from measurements of tumours irradiated 5 times per week for 3 weeks with daily doses of 70 rads of 15 MeV neutrons. The effects of this treatment are very similar to those obtained with 200 rads per day of 300 kV X-rays, although with respect to growth delay the data indicate that a treatment with 200 rads per day of 300 kV X-rays is slightly more effective.

In Fig. 5 results presented are obtained from tumours irradiated with 100 rads of 15 MeV neutrons per day. The data were measured only up to the end of the treatment period of three weeks. After the end of this treatment the tumours continued to decrease in volume and the yield of the cell dispersion technique was too low to obtain accurate data of the fractions of clonogenic cells. In the second and third week after the treatment most of the animals died presumably due to damage to the

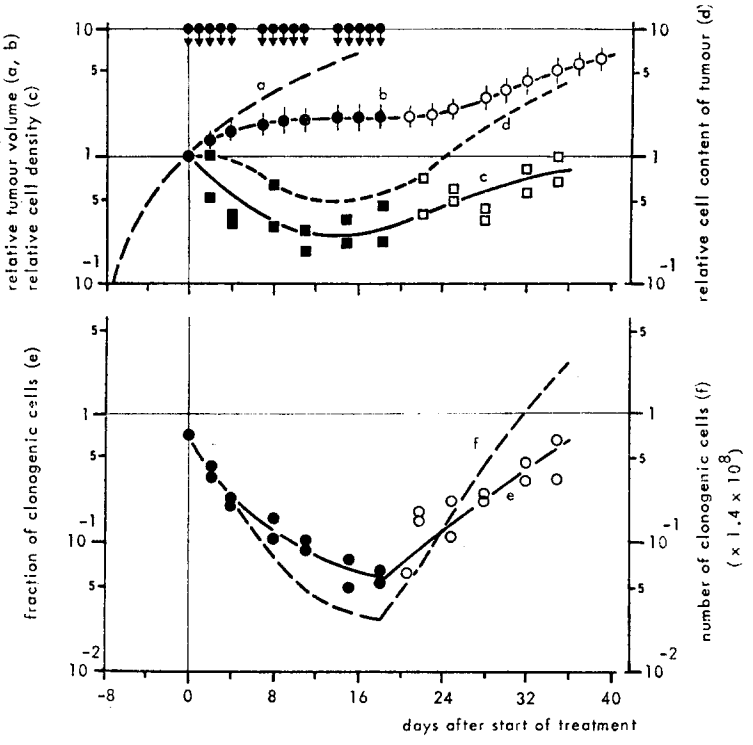


*Fig.* 3.
*Changes of tumour volume, numbers of cells and numbers of clonogenic cells in experimental R-1 rhabdomyosarcomas treated five times a week for three weeks with* 50 *rads of* 15 *MeV neutrons.*
*For description of curves a to f and symbols compare Fig.* 1.

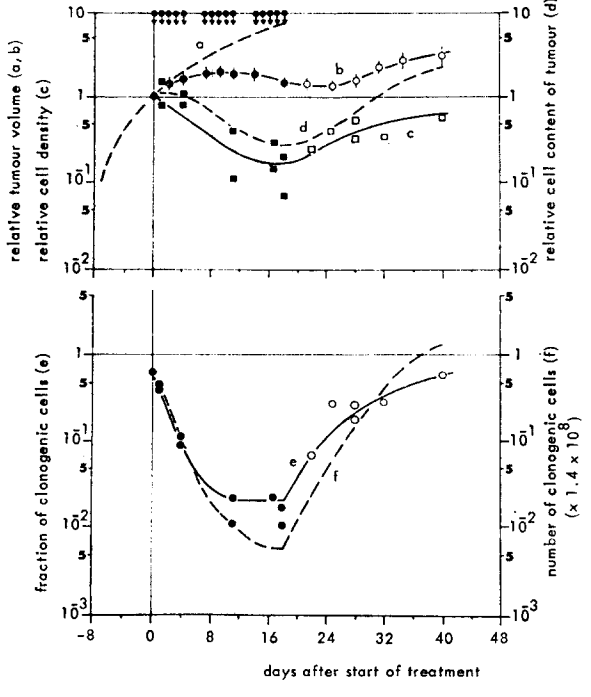


*Fig.* 4.
*Changes of tumour volume, numbers of cells and numbers of clonogenic cells in experimental R-1 rhabdomyosarcomas treated five times a week for three weeks with* 70 *rads of* 15 *MeV neutrons.*
*For description of curves a to f and symbols compare Fig.* 1.

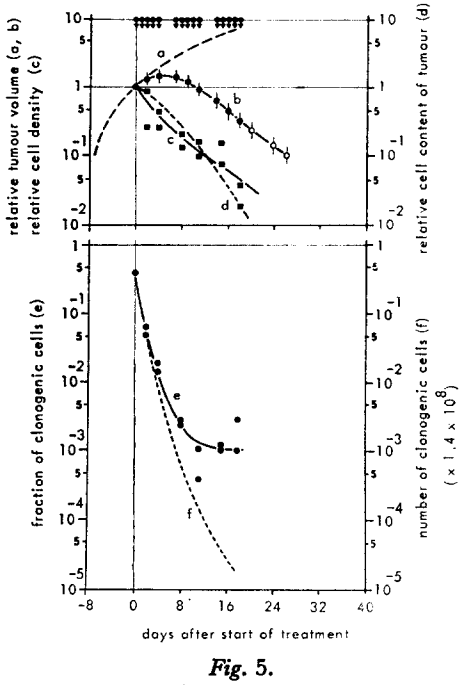


*Fig.* 5.
*Changes of tumour volume, numbers of cells and numbers of clonogenic cells in experimental R-1 rhabdomyosarcomas treated five times a week for three weeks with* 100 *rads of* 15 *MeV neutrons.*
*For description of curves a to f and symbols compare Fig.* 1.

*Table 1. Characteristics of cellular and volume responses of R-1 rhabdomyosarcomas irradiated five times per week for three weeks with different daily doses of 300 kV X-rays or 15 MeV neutrons*

| | daily dose fraction | | | | |
|---|---|---|---|---|---|
| | 300 kV X-rays | | 15 MeV neutrons | | |
| | 200 rads | 300 rads | 50 rads | 70 rads | 100 rads |
| 1. Fraction of clonogenic cells in tumour after first dose | 0·61±0·10 | 0·40±0·10 | 0·73±0·12 | 0·64±0·09 | 0·40±0·08 |
| 2. Ratio of numbers of clonogenic cells in tumour immediately after and before treatment | $8\times10^{-3}$ | $4\times10^{-5}$ | $3\times10^{-2}$ | $6\times10^{-3}$ | $2\times10^{-5}$ |
| 3. Ratio of minimum volume of tumour and pre-irradiation volume | 1·0 | 0·25 | 2·0 | 1·4 | <0·1 |
| 4. Mean doubling time of number of clonogenic cells during first two weeks after treatment | 2·7 days | 1·9 days | 2·7 days | 2·3 days | — |
| 5. Time interval from start of treatment till regrowth to pre-irradiation volume | 27 days | 44 days | — | 23 days* | — |
| 6. Volume doubling time of recurring tumour from the time the pre-irradiation volume is attained | 10 days | 11 days | — | 8 days* | — |
| 7. Growth delay | 33 days | 51 days | 17 days | 23 days | — |

*Extrapolated value.

blood forming tissues. As noted earlier, the animals received a total body dose which was approximately half the dose to the tumour due to the fact that sufficient shielding could not be used because of the low output of the neutron generator employed. More complete data will be obtained with a new generator which is presently being installed.

A summary of the characteristic values, which can be derived from the data, has been presented in Table 1.

(b) *Tumour volume changes after irradiation with daily doses of 300 kV X-rays and 15 MeV neutrons applied for different treatment periods*

In Fig. 6A, results are presented of tumour volume measurements during and after treatments of tumours with daily doses of 400 rads of 300 kV X-rays, administered five times per week for one, two, three and four weeks respectively. During the first week of treatment the tumours continued to grow and reached a volume equal to about 1·8 times the volume present at the start of the treatment. In case the treatment is applied for only one week, a small volume decrease is observed during the first week after the end of the treatment and growth is resumed in the second week after treatment. Growth delay, measured as the difference in time interval required by treated and untreated tumours to attain twice the volume at the time of irradiation, is approximately 16 days.

If the treatment is applied for two weeks, a distinct volume regression is observed during the second week and during about two weeks after the treatment. Growth is resumed during the third week after the end of the treatment. Growth delay can be calculated at 41 days. It is of interest to note that the recurrent tumours grow significantly slower than control tumours of the same size. At day 36 the mean volume is equal to the volume at the start of the treatment and at 45 days it has reached twice that volume. Thus the volume doubling time of the irradiated tumour during this period of recurrence is about 9 days as compared to between 4 and 5 days for untreated tumours of the same volume.

After treatment with 400 rads per day for three weeks the tumours become very small and cannot be measured accurately for about three weeks. A recurrence is observed, however, starting at about 50 days after the start of the treatment. These tumours attain a mean volume equal to that present at the start of the treatment at day 72 and they reach twice this volume about 12 days later.

After treatment with 400 rads per day for four weeks, the tumours disappear completely

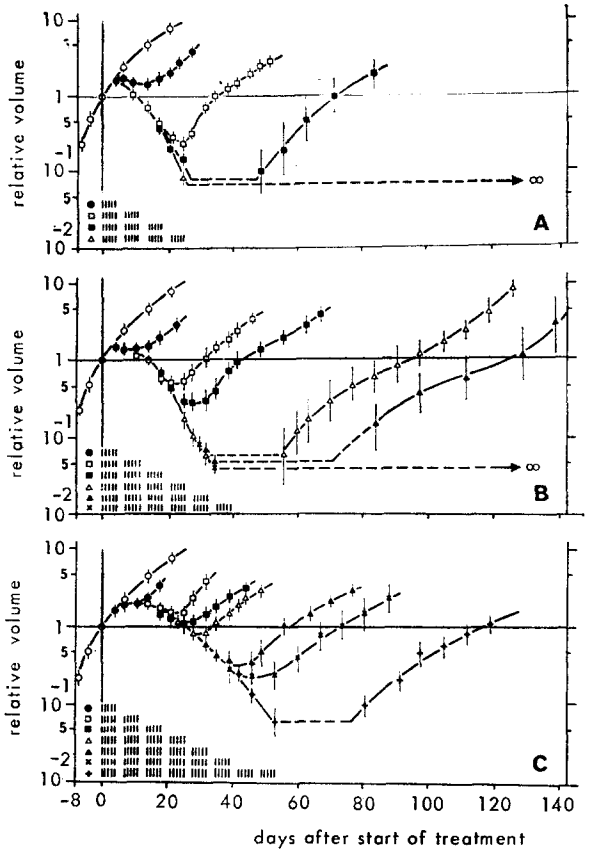


*Fig.* 6.

*Growth curves of R-1 rhabdomyosarcomas irradiated five times a week for different numbers of weeks with (A) 400 rads, (B) 300 rads and (C) 200 rads of 300 kV X-rays respectively. The vertical lines after the different symbols in the left corner indicate the days at which radiation was administered. Volumes are given relative to tumour volume at start of the treatment (mean value 0·8 cm³).*

*Vertical lines through points indicate standard errors of mean of at least five tumours.*

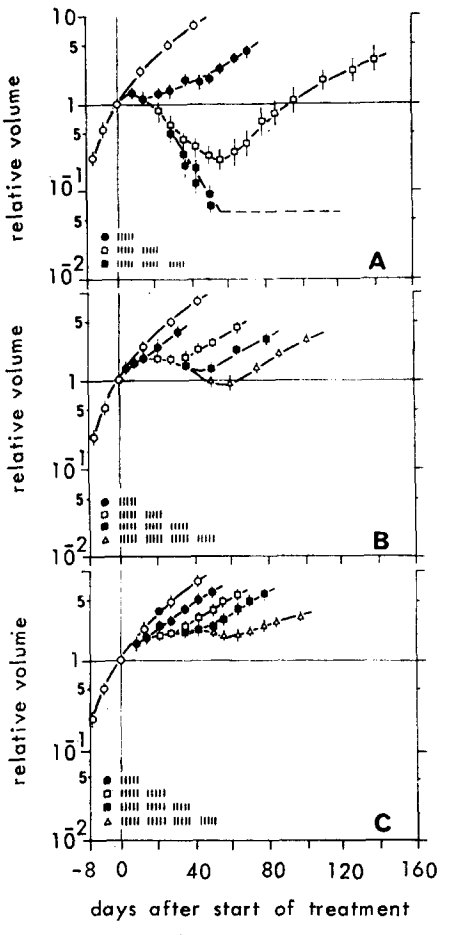


*Fig.* 7.

*Growth curves of R-1 rhabdomyosarcomas irradiated five times a week for different numbers of weeks with (A) 100 rads, (B) 70 rads and (C) 50 rads of 15 MeV neutrons respectively. The vertical lines after the different symbols in the left corner indicate the days at which radiation was administered. Volumes are given relative to tumour volume at start of the treatment (mean value 0·8 cm³).*

*Vertical lines through points indicate standard errors of mean of at least five tumours.*

and do not recur at all, as indicated by the ∞ symbol in Fig. 6A.

In Fig. 6B, results are presented of volume measurements during and after treatments of tumours with 300 rads of 300 kV X-rays per day, five times per week, for different numbers of weeks. All tumours recurred after five weeks of treatment. while after six weeks of treatment no recurrences were observed. After treatments of two weeks or more, recurrent tumours have smaller growth rates than untreated tumours of equal volume.

In Fig. 6C, results are presented of volume measurements during and after treatments of tumours with 200 rads of 300 kV X-rays per day, five times per week, for different numbers of weeks. Even after eight weeks all tumours recurred. Attempts to obtain cures by still longer treatments failed because the animals died due to the anaesthesia applied daily for prolonged periods.

In Fig. 7A, volume variations are presented of tumours irradiated five times per week for one, two and three weeks respectively with 100 rads of 15 MeV neutrons. After three weeks of treatment all animals died within 60 days after the end of the irradiation. This death is presumably due to bone marrow damage resulting from the fact mentioned earlier that sufficient shielding could not be provided and as a consequence the animals received a total body dose which was equal to about half the dose to the tumours.

In Fig. 7B and C, volume variations are presented for treatments with daily doses of 70 rads per day and 50 rads per day of 15 MeV neutrons respectively. Treatments for more than 4 weeks have not been applied because of the attendant risk of intercurrent death of the animals.

## DISCUSSION

(a) *The relation between tumour volume changes and variations of cellular parameters*

The results presented in Fig. 1 to 5 show that, during and after three weeks of treatments with different daily doses, tumour volume changes are much smaller than variations measured at the cellular level. Comparison of the data of lines 2 and 3 of Table 1, derived respectively from curves f and b of the figures, demonstrates that, for instance, the treatment with daily doses of 200 rads for three weeks reduces the number of cells capable of unlimited proliferation by a factor of $8 \times 10^{-3}$, while the volume first continues to increase during treatment, later decreases to the pre-irradiation value and growth is resumed during the second week after the end of the treatment. This difference between the extent of cell reproductive death and of volume changes is observed for all treatments where both values have been measured. In the case of 100 rads of 15 MeV neutrons per day, the volume decreased to a value which could no longer be measured, while recurrence could not be observed due to intercurrent death of the animals. A number of factors contribute to this difference between changes at the macroscopic level and the cellular level. Some of these factors have been discussed by Lajtha and Oliver [38] but the present results show that the phenomena occurring at the cellular level during regression and recurrence of a tumour as a result of a fractionated treatment are more complex than has been anticipated. Firstly, cells which have lost the capacity for unlimited proliferation, may divide a few times before proliferation is stopped. In addition, although division is stopped, tumour volumes may continue to increase due to an increase in mean cell volume. Furthermore loss of cells from the tumour which should give rise to tumour volume regression appears to be a rather slow process [2]. These three factors delay volume regression for a prolonged interval during which the cells that have retained the capacity for unlimited proliferation may repopulate the tumour. This repopulation can occur at a high rate, as can be deduced from data concerning cell cycle times and cell loss factors measured by autoradiographic techniques, which indicate that the rate of cell production is increased after irradiation with single doses of 1000 and 2000 rads of X-rays [2]. This increase was shown to be due to a shortening of the cell cycle time from about 20 hr to about 12 hr, while the growth fraction changes were relatively small [2]. Whether this phenomenon plays a part during and after fractionated irradiation has not yet been investigated with autoradiography, but from the results obtained with respect to changes of numbers of clonogenic cells during and after the end of different treatments it can be inferred that indeed the rate of proliferation of cells in the irradiated tumour might be increased as compared with unirradiated tumours. As noted in the previous section the rate of decrease of the number of clonogenic cells, represented by the slopes of curves f of Fig. 1–5, diminishes during the course of the treatment. In the case of 200 rads of X-rays per day a decrease in the number of clonogenic cells by a factor of 10 can be derived from curve f for the first week of treatment, by a factor of 6 for the second week, and a factor of only 2 for the third week. In the case of 300 rads of X-rays per day the decrease of the

*Table* 2. *Decrease of numbers of clonogenic cells during treatment with* 300 *kV X-rays or* 15 *MeV neutrons, five times per week for three weeks*

| Doses given | Ratio of numbers of clonogenic cells before and after treatment period of | | |
|---|---|---|---|
| | first week | second week | third week |
| 300 kV X-rays | | | |
| 200 rads/day, 5d/week | 10 | 6 | 2 |
| 300 rads/day, 5d/week | 50 | 60 | 8 |
| 15 MeV neutrons | | | |
| 50 rads/day, 5d/week | 5 | 4 | 2 |
| 70 rads/day, 5d/week | 10 | 8 | 2 |
| 100 rads/day, 5d/week | 100 | 70 | 7 |

number of clonogenic cells is equal to a factor of about 50 in the first week, a factor of about 60 in the second week, but only a factor of about 8 in the third week. As shown in Table 2 similar decreases in effectiveness of the daily dose with increasing treatment time are also observed during treatments with 15 MeV neutrons. This decrease in the net effect of the irradiation might be due to three factors:

(1) The presence of severely hypoxic cells, which might become predominant as irradiation, selectively eliminates the well-oxygenated cells (compare factor 6 of the introduction).

(2) Survival of those cells in a tumour which are most resistant to small doses of radiation applied at 24 hr intervals. This resistance might be due to a large $D_{37}$, to a large $N$ corresponding to a large capacity for repair of sub-lethal damage, or to an induced synchrony of the cells in the generation cycle as a consequence of the irradiation at 24 hr intervals (compare the factors 1–5 of the Introduction).

(3) Rapid proliferation of surviving cells during intervals between daily doses (compare factor 8 of the Introduction).

The first of these three factors has been investigated in a series of experiments concerning changes in fractions of anoxic cells to be reported in detail separately. In Table 3 preliminary data are tabulated concerning fractions of anoxic cells in R-1 rhabdomyosarcomas measured during three weeks of treatments with 300 rads of 300 kV X-rays daily. These data have been derived by the application of test doses of 1200 rads respectively to tumours in living animals with a fraction of hypoxic cells and to animals killed 10 min before irradiation so that all cells have become hypoxic. As discussed extensively by others [19, 22–24, 26, 28, 31], the ratio of the fractions of surviving cells measured for both conditions provides a measure of the fraction of anoxic cells. For the data presented in Table 3 tumours were used which had been irradiated five days per week for different time intervals and assays by the *in vitro* plating technique were performed after the test doses were given. The results must be considered as preliminary and differences of less than 50% are not significant. This is due to the fact that the analysis of fractions of anoxic cells by the application of additional test doses to tumours in which the fractions of clonogenic cells are already low due to previous treatments, presents serious technical difficulties as discussed in Part I of this series. Nevertheless, the results are sufficiently consistent to show that the fraction of anoxic cells does not increase to close to 100%, as would be expected if the daily treatments were to eliminate all well-oxygenated cells preferentially. It can be concluded that reoxygenation of previously anoxic cells occurs in the R-1 rhabdomyosarcoma to a considerable extent. Consequently, the decrease in the effectiveness of the daily irradiation demonstrated by the decreasing slope of curve f of Fig. 2 during the three weeks of treatment cannot be explained by a large increase of the fraction of anoxic cells. In the cases of 200 rads of 300 kV X-rays and of different doses of 15 MeV neutrons the selective elimination of oxygenated cells should be of less importance than with 300 rads per day of 300 kV X-rays because of the smaller dose employed and in the case of 15 MeV neutrons because of the small oxygen enhancement ratio.

*Table 3. Fraction of anoxic cells in R-1 rhabdomyosarcomas during treatment with daily doses of 300 rads of 300 kV X-rays*

| Days after start of treatment | Accumulated in rads | Mean fraction of clonogenic cells in tumour | Fraction of anoxic cells |
|---|---|---|---|
| 0 | 0 | 100 | 0·15 |
| 3 | 900 | 0·07 | 0·17 |
| 5 | 1500 | 0·04 | 0·08 |
| 9 | 2100 | 0·005 | 0·22 |
| 12 | 3000 | 0·002 | 0·40 |
| 16 | 3600 | 0·002 | 0·30 |
| 18 | 4200 | 0·002 | 0·30 |
| 19 | 4500 | 0·002 | 0·16 |

With respect to the second possibility to explain the decrease in effectiveness during the third week of treatment on the basis of a low radiosensitivity of the cells, some conclusions can be derived from a comparison of results obtained with 300 kV X-rays and 15 MeV neutrons. Survival curves of cells irradiated with 15 MeV neutrons show a smaller extrapolation number as compared with X-rays, indicating that relatively less repairable damage is produced [9]. In addition, smaller differences of the radiosensitivity as a function of age in the cell cycle are observed with high-LET radiations as compared with low-LET radiations [7, 39, 40]. Consequently one would expect that if selective killing of cells with a small capacity for repair or synchronization of cells in a resistant phase of their cycle

due to daily irradiation played a part in diminishing the effectiveness in the last week of the treatment, this phenomenon would be at least partly eliminated in the case of 15 MeV neutrons. However, for the case of daily doses of 70 rads of 15 MeV neutrons, curve f of Fig. 4 shows decreases of the numbers of clonogenic cells by factors of 10, 8 and 2, respectively, in the first, second and third week of the treatment. These values are very similar to the factors derived for daily doses of 200 rads of 300 kV X-rays. Consequently, although selective killing of cells with a low extrapolation number and synchronization of cells in a resistant phase of the cycle at 24 hr intervals cannot be ruled out, most of the decrease in effectiveness during the third week must be due to the third factor mentioned, namely rapid proliferation of clonogenic cells, which in the 24 hr intervals partly compensates for the cells rendered incapable of unlimited proliferation by each daily dose fraction. This conclusion is supported by the fact, discussed in the next section, that after the treatment the numbers of clonogenic cells increase very rapidly.

(b) *Growth characteristics of recurring tumours and their constituent cells*

The treatment schedules for which tumour volume changes as well as the variations of numbers of clonogenic cells have been measured were not sufficient for tumour cures to be achieved. As discussed in Part I of this series, the tumours with a mean volume of 0·8 $cm^3$ contained an average of $1{\cdot}4\times10^8$ clonogenic cells. Thus even after a treatment with 300 rads of X-rays per day for three weeks, which reduces the number of clonogenic cells by a factor of $4\times10^{-5}$, about $5{\cdot}6\times10^3$ clonogenic cells are still present and cures would not be expected.

The data presented by the open symbols of Fig. 1–5 pertain to tumours which recur after three weeks of treatment. At various time intervals after the end of the treatment volume changes and cellular reproductive integrity were assayed. The curves b of Fig. 1–5 show that the volumes of the tumours may continue to decrease for a time interval of at least two weeks after the end of the treatment. The extent of this decrease clearly depends on the dose administered.

In contrast to the tumour volume and the total numbers of cells in the tumours, the fractions of clonogenic cells (curve e) and the numbers of clonogenic cells (curves f) start to increase very shortly after the end of the treatments. The increase in the fraction of clonogenic cells (curves e) is due to production of new clonogenic cells and removal of killed cells, while for the calculation of the curves f the latter factor has been eliminated. A measure of the rate of increase of the number of clonogenic cells during the first two weeks after treatments with different regimes can be derived from curves f by calculation of the mean doubling times. The values, which range from 1·9 to 2·7 days, have been given in line 4 of Table 1. These values are smaller than the corresponding doubling times of the numbers of clonogenic cells in unirradiated tumours of equal volume, which range from 3·5 to 5 days. This difference demonstrates that changes which take place in irradiated tumours may cause an increased rate of production of cells, which partly compensates for the damage produced by daily irradiation. As a consequence the tumours recur earlier than would be expected on the basis of the numbers of cells which have retained the capacity for unlimited proliferation and the growth rate of unirradiated tumours. In the three weeks of treatment with 300 rads of X-rays per day, for instance, the number of clonogenic cells in the tumour is reduced by a factor of $4\times10^{-5}$. The tumour volume shrinks only to a value equal to 0·25 times the volume at the start of the treatment. Although the volume doubling time during regrowth varies from 6 to 11 days, the clonogenic cells repopulate the tumour with a doubling time of about 2 days and cause an early recurrence. A similar phenomenon has been observed after single doses and has been discussed in detail in Part I of this series [1].

It is of interest to note further that during the third week after the end of the treatment, coinciding with resumption of tumour growth, the rate of increase of the numbers of clonogenic cells diminishes. As shown earlier, the volume doubling times of tumours, which have regrown to their pre-irradiation size, are larger than the value of 4 to 5 days measured for unirradiated tumours (line 6 of Table 1). This may be due to two factors, namely an increase of the cell generation cycle and a decrease of the growth fraction [2]. The latter factor would imply that the cells which proliferate in the recurrent tumour have sustained non-lethal damage of the type which gives rise to small colony formation *in vitro* [35, 36, 41]. Measurements of cell cycle parameters and cell loss factors in tumours recurring after fractionated treatments are required, however, to evaluate the relative importance of these factors.

From a comparison of volume doubling

times of untreated and treated tumours it can be concluded that the growth delays derived for various treatments depend on the tumour volume employed for assessment of this parameter. For instance after daily doses of 300 rads of 300 kV X-rays, applied 5 times per week for three weeks, the tumour regrows to the pre-irradiation volume in 44 days (line 5 of Table 1). If regrowth to twice the pre-irradiation volume is used for assessment of growth delay, a value of 51 days is obtained because 55 days are required to reach that volume and the doubling time of 4 days for unirradiated tumours must be subtracted (line 7 of Table 1). If still larger volumes were used as criterium for assessment of growth delay, the value would be even larger. It is evident that growth delay is not an unambiguous measure of the effectiveness of a given treatment. It is clear that the numbers of clonogenic cells present in the tumour at the end of the treatment are of primary importance with respect to the probability of tumour cure and recurrence. However, if no cure is attained the time interval required by the tumour to regrow to a given volume depends on the post-irradiation proliferation cell kinetics and cannot be predicted on the basis of this parameter alone [42].

(c) *Changes in tumour responses to fractionated irradiation during protracted treatments*

As discussed in the previous paragraphs, the net effect with respect to the decrease of the numbers of clonogenic cells produced by daily treatments of tumours with small doses of 300 kV X-rays or 15 MeV neutrons, diminishes especially during the third week. This result is in agreement with the growth delay data presented in Fig. 6. After irradiation with daily doses of 200 rads of 300 kV X-rays, for instance, two weeks of treatment induces a growth delay of 23 days, three weeks of treatment causes a delay of 32 days and four weeks of treatment a delay of 39 days. Calculation of the differences shows that the additional growth delay induced by the third and fourth week of treatment amounts to only 9 and 7 days respectively. The fifth week of treatment is much more effective however, inducing an additional growth delay of 25 days. As discussed earlier, the relatively small effect of the daily irradiation with 200 rads of X-rays per day during the third week must be attributed mainly to an increased rate of proliferation of the clonogenic cells. No measurements of the decrease of numbers of clonogenic cells during the fourth and fifth week of treatment have yet been made, but it might be speculated that as the treatment with 200 rads of X-rays per day is continued beyond four weeks, the rate of production of clonogenic cells diminishes again causing an increase in effectiveness of the fifth week of treatment. This might for instance be due to an accumulation of non-lethal damage, causing a decrease in the growth fraction (factor 9 of the Introduction). The growth curves of Fig. 6C for longer irradiation show that the additional growth delay produced by the 6th, 7th and 8th week of treatment with 200 rads of X-rays per day are also much larger as compared with the effect of the third and fourth week of this treatment. Thus the increased proliferation rate of clonogenic cells appears to be restricted to the third and fourth week of the treatment.

A similar but less pronounced variation in the response of the tumours with respect to growth delay can be noted in the case of daily doses of 300 rads of X-rays per day (Fig. 6B). The additional growth delay induced by the third week is smaller as compared with either the second or the fourth and fifth week. In addition to growth-delay data, consideration of the decrease in the numbers of clonogenic cells also indicates that after a decline during the third week the net effect of daily irradiations with 300 rads of X-rays per day increases during the fourth and fifth week. As shown in Table 2, the decrease in the number of clonogenic cells during the third week amounts to a factor of 8. As noted earlier at the end of the third week the tumours contained an average of $5 \cdot 6 \times 10^3$ clonogenic cells. After six weeks of treatment with 300 rads of X-rays per day, no tumour recurred however. It may be assumed that after the end of 6 weeks of treatment the mean number of clonogenic cells was reduced to less than one, i.e. by a factor of at least 5600. This assumption is supported by the fact discussed in Part I of this series that for single doses the TCD90 value extrapolated from the cell survival curves was very close to the measured TCD90 [1]. It may be concluded that during each of the fourth, fifth and sixth weeks of treatment the numbers of clonogenic cells have been reduced by a mean factor of about 18, which is much larger than the factor of 8 derived from measurements during the third week.

In the case of 400 rads of X-rays per day a similar change in the effectiveness of daily irradiation cannot be detected because after four weeks of treatment with this daily dose no tumours recurred.

In the cases of treatments with 15 MeV neutrons a possible variation in the response

with time, if present, could not be measured since continuation of the treatments was not possible due to the low output of the neutron generator which did not allow sufficient shielding of the animals. More data are obviously required to evaluate in detail the causes of the variation of the effectiveness of daily irradiation during protracted treatments.

(d) *Relative effectiveness of different fractionated treatments with* 300 *kV X-rays and* 15 *MeV neutrons compared to single exposures*

In Fig. 1–5 results of irradiations of R-1 rhabdomyosarcomas with different daily doses of X-rays and 15 MeV neutrons have been presented as a function of the time interval after the start of the treatment. In order to assess the relative effectiveness of various treatments per unit dose, the data with respect to variations of numbers of clonogenic cells in the tumours, derived from the curves f of Fig. 1–5, have been presented in Fig. 8 as a function of the accumulated dose. The points shown in the curves were derived from the corresponding values of the curves f at the end of each week of treatment. For instance, the closed square at 2000 rads corresponds to the value of 0·016 derived from curve f of Fig. 1 for the end of two weeks of treatment of tumours with five daily doses of 200 rads of X-rays per week. In Fig. 8 the survival curves obtained for cells assayed after single exposures of tumours in living animals have been included (curves a and b) [1]. The solid lines 1 and 1′ have been drawn through the points representing numbers

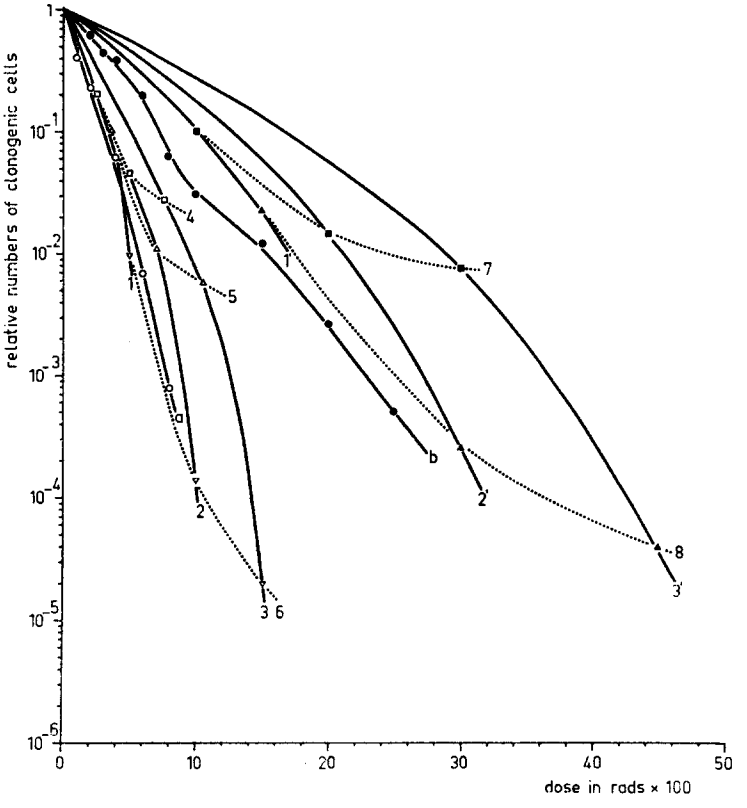


*Fig.* 8.

*Relative numbers of clonogenic cells present at the end of various treatments of R-*1 *rhabdomyosarcomas with* 300 *kV X-rays and* 15 *MeV neutrons, relative to the numbers present at the start of the treatment, as a function of the accumulated dose.*

*Curves a and b: Single doses of* 15 *MeV neutrons and* 300 *kV X-rays respectively.*

*Curves* 1 *and* 1′*: Five equal fractions of* 15 *MeV neutrons and* 300 *kV X-rays respectively, overall treatment time* 5 *days.*

*Curves* 2 *and* 2′*: Ten equal fractions of* 15 *MeV neutrons and* 300 *kV X-rays respectively, overall treatment time* 12 *days.*

*Curves* 3 *and* 3′*: Fifteen equal fractions of* 15 *MeV neutrons and* 300 *kV X-rays respectively, overall treatment time* 19 *days.*

*Dotted curves* 4, 5 *and* 6 *connect points obtained with equal daily doses applied for* 1, 2 *and* 3 *weeks. Dose fractions of* 50, 70 *and* 100 *rads of* 15 *MeV neutrons for curves* 4, 5 *and* 6 *respectively.*

*Dotted curves* 7 *and* 8 *connect points obtained with equal daily doses applied for* 1, 2 *and* 3 *weeks. Dose fractions of* 200 *and* 300 *rads of* 300 *kV X-rays for curves* 7 *and* 8 *respectively.*

of clonogenic cells in the tumours relative to the numbers present at the start of the treatment, obtained after one week of treatment with various doses of 15 MeV neutrons and 300 kV X-rays respectively. Similarly the curves 2 and 2′ were drawn through points corresponding to two weeks of treatment and 3 and 3′ through points corresponding to three weeks of treatment. All curves show increasing slopes with increasing total doses accumulated in a given number of weeks, i.e. with increasing daily doses. The shapes of curves a and b for single exposures to 15 MeV neutrons and 300 kV X-rays, respectively, are at larger doses determined mainly by the presence of anoxic cells [1].

From the curves of Fig. 8 it is possible to derive factors for the ratios of the doses required to attain a given effect with five, ten or fifteen fractions, given in a specified overall treatment time relative to single doses required for the same effect. These values have been summarized in Table 4. It can be concluded that for X-rays as well as for neutrons the factors by which the single doses must be multiplied in order to derive the total doses of fractionated irradiation required for the same reduction of clonogenic cells increase with increasing number of fractions. The factors for low levels of effect, i.e. decreases of the numbers of clonogenic cells to 10% and 1%, are larger for 300 kV X-rays as compared with 15 MeV neutrons, but at the 0·1% level the factors for equal numbers of fractions are approximately the same for both radiations. These values represent the net effect of a variety of factors. Some of these factors including repair of sub-lethal damage, the presence of anoxic cells and reoxygenation have a smaller influence in the case of 15 MeV neutrons as compared with 300 kV X-rays. Proliferation of cells during protracted treatments, however, is presumably similar during corresponding treatments with both types of radiation. A more detailed analysis of these data and their correlation with doses required for cures will be given in a subsequent paper.

The dotted lines in Fig. 8 connect the points which have been obtained for treatments of 1, 2 and 3 weeks with equal daily doses respectively. All curves for the fractionated exposures show a decrease of the slope with increasing dose which is most pronounced in the regions corresponding to the third week of treatment. As discussed earlier this decrease in effectiveness of subsequent weeks of treatment is mainly caused by the increased rate of proliferation of clonogenic cells in irradiated tumours and little by an increase of fractions of anoxic cells.

From the data presented in Fig. 6 and 7,

*Table* 4. *Ratio of fractionated dose to single dose required for equal effects of* 300 *kV X-rays and* 15 *MeV neutrons on R-*1 *rhabdomyosarcoma*

| Treatment regime | Decrease of numbers of clonogenic cells to 10% | to 1% | to 0·1% |
|---|---|---|---|
| 300 kV X-rays | | | |
| 5 fractions in 5 days | 1·4 | 1·1 | — |
| 10 fractions in 12 days | 1·8 | 1·4 | 1·2 |
| 15 fractions in 19 days | 2·3 | 1·9 | 1·6 |
| 15 MeV neutrons | | | |
| 5 fractions in 5 days | 1·1 | 0·9 | — |
| 10 fractions in 12 days | 1·2 | 1·3 | 1·2 |
| 15 fractions in 19 days | 1·7 | 1·7 | 1·6 |

| | Growth delay 20 days | 80 days |
|---|---|---|
| 300 kV X-rays | | |
| 200 rads/day, 5d/week | 0·8 | 1·3 |
| 300 rads/day, 5d/week | 0·9 | 1·2 |
| 400 rads/day, 5d/week | 1·2 | 1·3 |
| 15 MeV neutrons | | |
| 50 rads/day, 5d/week | 1·3 | — |
| 70 rads/day, 5d/week | 1·4 | — |
| 100 rads/day, 5d/week | 0·8 | — |

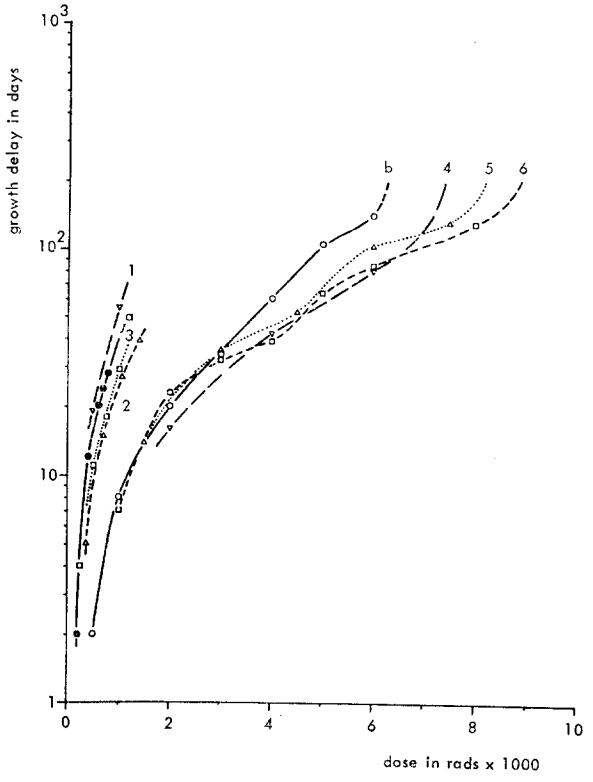


*Fig.* 9.

*Tumour growth delay of R-1 rhabdomyosarcomas as a function of accumulated doses of* 15 *MeV neutrons or* 300 *kV X-rays.*

*Curve a: Single doses of* 15 *MeV neutrons* [1].

*Curve b: Single doses of* 300 *kV X-rays* [1].

*Curves* 1, 2 *and* 3*: Daily doses of* 100, 70 *and* 50 *rads of* 15 *MeV neutrons respectively, applied five times a week for different numbers of weeks.*

*Curves* 4, 5 *and* 6*: Daily doses of* 400, 300 *and* 200 *rads of* 300 *kV X-rays respectively, applied five times a week for different numbers of weeks.*

values for tumour growth delay induced by different treatments can be derived. These values are presented as a function of the dose in Fig. 9. Curves a and b of this figure represent growth delays induced by single exposures [1]. From the curves of Fig. 9 the ratios of doses required in fractionated treatments relative to single doses of the same radiation for induction of a given growth delay can be derived. A growth delay of 20 days is obtained with a single dose of 2000 rads (curve b) while according to curve 4 for 400 rads per day a dose of 2400 rads is required for the same delay. From these values a ratio of 1·2 can be calculated for the dose required with the fractionated treatment with 400 rads of 300 kV X-rays per day relative to the single dose. Further numerical values of the ratios of doses required with fractionated treatments relative to single doses are presented in Table 4. For 300 kV X-rays, values are given for a relatively short delay of 20 days and for a long growth delay of 80 days. For 15 MeV neutrons, values of the relative effectiveness of fractionated treatments cannot be calculated for a delay of 80 days because such long delays could not be induced with the low yield of the neutron generator employed. It is important to note that the numerical values presented in Table 4 for the dose ratios with respect to reduction of the numbers of clonogenic cells cannot be compared directly with the dose ratios required for a given growth delay because the former values correspond to effects produced in equal treatment intervals, while the values for growth delay could only be derived by comparison of doses given in specified daily doses but not for equal total treatment times.

Figure 9 shows that the shapes of the curves are different. As discussed in the preceding paragraph the effectiveness of a given dose fraction may change during the course of the treatment and this is reflected in the differences between the slopes of curves 4, 5 and 6. Consequently the effectiveness relative to the single dose depends on the growth delay considered.

It is finally of interest to discuss briefly the

relation between the total doses of 300 kV X-rays required to obtain tumour cures with different fractionation schemes. From the limited data available it is not possible to derive accurately the doses required for a proportion of 90% of tumours cured (TCD90). For the single dose treatment with 300 kV X-rays this value is 6500 rads [1]. With 400 rads of 300 kV X-rays per day, applied five times per week, all ten tumours treated were cured after a total dose of 8000 rads in four weeks of treatment. From the shape of curve 4 of Fig. 9, indicating that at 6000 rads the growth delay is only about 80 days, it can be deduced that the TCD90 is presumably not much smaller than 8000 rads, probably between 7000 rads and 8000 rads. Similarly the TCD90 for 300 rads of X-rays daily may be estimated to be found between 8000 and 9000 rads, while for 200 rads per day this value is presumably in excess of 9000 rads. It can be concluded that the TCD90 increases with decreasing daily dose fractions, i.e. with increasing numbers of fractions. This relation has been observed in experiments with animal tumours as well as with treatments of human tumours and the shape of the curve relating the doses required for cures with the number of fractions has been explained on the basis of the repair of sub-lethal damage [4, 8, 38, 43]. Discussions of this interpretation have frequently been based on simplified hypothetical survival curves, derived from multi-target models which fail to take into account the LET-distributions of X-rays and gamma-rays and the consequent initial negative slope of the survival curves. The exact shapes of mammalian cell survival curves in the low dose region have been measured for only a few cell systems however. The present investigations show that responses of the R-1 rhabdomyosarcoma to fractionated irradiation are complicated by reoxygenation of hypoxic cells and rapid repopulation of cells in the tumour through an increased proliferation rate observed especially during the third week of treatment. It is of interest to note for instance that for a relatively short growth delay of 20 days the relation between the total dose of X-rays required and the number of fractions is different from the relation for attaining cures. Figure 9 shows for instance that 400 rads per day is per unit dose the least efficient and 200 rads per day is per unit dose the most efficient treatment for inducing 20 day-growth delay. This might be due to an insufficient rate of reoxygenation in the case of 400 rads per day of X-rays. It can further be concluded from Fig. 9 that in the last part of the treatment, larger daily doses are more efficient per unit dose than small daily doses. This might in part be due to the fact that with large daily doses the increased proliferation rate has a relatively small effect, because a given total dose is administered in a shorter interval as compared with treatments with small daily doses. However, the relative importance of these factors cannot be evaluated from the limited data available.

(e) *RBE-values of* 15 *MeV neutrons relative to* 300 *kV X-rays*

From the curves of Fig. 8 it is possible to derive RBE-values of 15 MeV neutrons relative to 300 kV X-rays. Comparison of curves 1 and 1′ shows for instance that in order to obtain within one week a reduction of the number of clonogenic cells to 10% of the number at the start of the treatment, doses of 350 rads of 15 MeV neutrons and 1000 rads of 300 kV X-rays are required. From these values an RBE of 2·9 can be calculated. RBE-values corresponding to a reduction of the numbers of clonogenic cells to 10%, 1% and 0·1% in equal treatment periods have been summarized in Table 5. For comparison RBE-values derived for single doses have been included. These RBE-values depend on the fractionation regimes compared. The large RBE-values for a reduction of the numbers of clonogenic cells to 10% are at least partly due to the differences in slopes of the low dose regions of the survival curves for 15 MeV neutrons and 300 kV X-rays respectively. The RBE-values of about 3·0 for longer overall treatment times and large reductions of the number of clonogenic cells are the most important with respect to the assessment of possible advantages of 15 MeV neutrons for application in radiotherapy. These values are not significantly different from the value of 2·9 obtained for single doses at the 0·1% level. For single doses the relatively large value of 2·9 was due to the presence of anoxic cells. In the fractionated treatments these anoxic cells reoxygenate during daily intervals and the RBE of 2·9 to 3·0 obtained is due to differences in the extent to repair of sub-lethal damage (compare factor 2 of the Introduction). The RBE-values discussed must be compared with RBE-values for effects of similar fractionation schemes on normal tissues before possible advantages of fast neutrons can be evaluated [7, 8, 30, 44].

RBE-values presented in Table 5A have been derived by comparison of treatments which produce equivalent effects in equal overall treatment times. Such a comparison is not yet

*Table 5. RBE-values of 15 MeV neutrons relative to 300 kV X-rays for single and fractionated exposures of R-1 rhabdomyosarcomas in the rat*

| A. Reduction of numbers of clonogenic cells | | | |
|---|---|---|---|
| Treatments compared | RBE at 10% | RBE at 1% | RBE at 0·1% |
| 1. Single doses | 2·4 | 2·7 | 2·9 |
| 2. 5 fractions in 5 days | 2·9 | 3·4 | — |
| 3. 10 fractions in 12 days | 3·4 | 3·0 | 3·0 |
| 4. 15 fractions in 19 days | 3·3 | 3·0 | 2·9 |

| B. Growth delay of 20 days | |
|---|---|
| Treatments compared | RBE* |
| 1. Single doses | 3·3 |
| 2. 200 rads/day of 300 kV X-rays and 50 rads/day of 15 MeV neutrons | 2·3 |
| 3. 200 rads/day of 300 kV X-rays and 70 rads/day of 15 MeV neutrons | 2·2 |
| 4. 300 rads/day of 300 kV X-rays and 100 rads/day of 15 MeV neutrons | 3·4 |
| 5. 400 rads/day of 300 kV X-rays and 100 rads/day of 15 MeV neutrons | 4·3 |

*Derived from Fig. 9 as described in discussion.

possible for growth delay, because sufficient data are not available. Therefore RBE-values for growth delay given in Table 5 have been derived by comparison of specific treatments with daily doses and it should be noted that the overall treatment times were not exactly the same. The RBE-values for growth delay show large differences, which may be caused partly by differences in the effects of reoxygenation and repopulation [30, 44]. The RBE-values for growth delay are for small daily doses lower than RBE-values derived for reduction of the numbers of clonogenic cells to different levels. These small RBE-values might be due partly to a more rapid regrowth of the tumour after irradiation with small daily doses of 15 MeV neutrons as compared with doses of X-rays. The data presented in Table 1, line 6, do not support this explanation, however. More data are required for longer growth delays in order to assess the significance of these differences.

## CONCLUDING REMARKS

The results discussed in this paper have shown that the responses of this experimental rhabdomyosarcoma to treatments with relatively small doses of 300 kV X-rays and 15 MeV neutrons, applied 5 times a week for several weeks, are very complex. It was concluded that repair of sub-lethal damage, the presence of anoxic cells, their changing oxygenation status during treatment and proliferation of cells during the 24 hr interval all influenced the final result of the treatment. The effectiveness per unit dose of the daily irradiation with 300 kV X-rays was demonstrated to change during protracted treatment, while the effect of severely hypoxic cells was found to be largely eliminated in fractionated irradiations with small daily doses due to reoxygenation of hypoxic cells during the intervals. As a consequence, the lower oxygen enhancement ratio of 15 MeV neutrons relative to 300 kV X-rays does not contribute appreciably to a large RBE of 15 MeV neutrons for small daily doses, in contrast to the case of single doses. Since for similar fractionated treatments very little data are available for effects on normal tissues, no definitive evaluation with respect to a possible therapeutic advantage of 15 MeV neutrons can yet be given however.

The results presented in this paper suggest that in the case of X-rays and other low-LET radiations rather small daily doses are the most effective in producing damage to the tumours by doses which cause a tolerable level of damage to normal tissues. This might be due to the presence of resistant hypoxic cells which require rather long time intervals for reoxygenation. It might be speculated, however, that if this dictating influence of anoxic cells could

be eliminated, other fractionation schedules could be employed which would take advantage of differences between the tumour and normal tissues, e.g. with respect to cell cycle kinetics. It will be clear that the use of fast neutrons would provide the advantage of at least partly eliminating the requirement of treatment schedules with small daily doses. This would allow more flexibility and offers the prospect that treatments can be applied which can be adapted to various growth properties of different tumours and relevant normal tissues. In subsequent papers results will be reported of treatments of the rhabdomyosarcoma with unorthodox fractionation schedules.

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## SUMMARY

*Experiments have been carried out to evaluate responses of a rhabdomyosarcoma, transplantable in an inbred strain of rats to various daily doses of* 300 kV *X-rays and* 15 MeV *neutrons applied five times per week for different numbers of weeks. Tumour growth delay and loss of cell reproductive integrity during and after treatments were compared in order to gain an insight in the relative importance of various factors which determine the effectiveness of different treatment schedules, namely intracellular repair of sub-lethal damage, the presence of anoxic cells and their reoxygenation during intervals and repopulation of tumours through proliferation of surviving cells. The results show that tumour volumes change to a much smaller extent than the fractions of cells which have retained the capacity for unlimited proliferation. During treatments of three or more weeks the effectiveness of a given daily dose for the reduction of the numbers of clonogenic cells is shown to vary. After the end of treatments applied five times a week for three weeks with daily doses of* 50, 70 *or* 100 *rads of* 15 MeV *neutrons and* 200 *or* 300 *rads of* 300 kV *X-rays, the surviving cells proliferate with a doubling time of about* 2 *days as compared with* 4 *to* 5 *days before irradiation.* RBE-*values of* 15 MeV *neutrons for fractionated irradiations are shown to be dependent on the schedules compared.*

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# The Effect of Dimethylbenzanthracene on the Incorporation of [$^3$H] Thymidine into DNA of Rat Mammary Gland and Uterus

R. J. B. KING and DOROTHEA M. COWAN
*Departments of Hormone Biochemistry and Pathology, Imperial Cancer Research Fund, Lincoln's Inn Fields, London W.C.2., England*

## INTRODUCTION

THE CARCINOGENIC effect of dimethylbenzanthracene (DMBA) on rat mammary gland is well documented [1] but the mechanism by which this occurs is unknown. DMBA prolongs the S phase of the cell cycle of mouse hair follicoles [2] and other authors have reported inhibitory effects on DNA synthesis [3–5].

In view of this it was of interest to study the effect of DMBA on DNA synthesis in the mammary gland. As ovarian hormones influence tumorigenesis in the mammary gland [1], the effect of these on DNA synthesis has also been studied.

## METHODS

*Animals*

Virgin female Sprague–Dawley rats were acclimatised to a cycle of 12 hr light and 12 hr dark with handling 2–3 hr after the beginning of the light period.

*Administration of chemicals*

In all cases, these were given 3 hr after the beginning of the light period. [$^3$H] thymidine (s.a. 3–5C/mmole, Radiochemical Centre, Amersham) was injected (intra peritoneal 1 μc/g. body weight) on the 50th day of life, 22 hr before death. DMBA or benzanthracene (30 mg in 2 ml corn oil) were given by stomach tube 24 hr before the [$^3$H] thymidine. Oestradiol injections were given in 0·2 ml corn oil s.c. in a mid-dorsal position between glands 3 and 4.

*Removal of mammary glands*

The whole pelt was removed and fixed in Bouin's fluid for 24 hr to visualise the glands. The majority of experiments were carried out on tissue from the centre of the gland which consisted of ducts and side buds. In experiments where only the terminal buds were used, only the large club shaped buds at the periphery of the gland, opposite the nipple, were used. It was sometimes necessary to combine buds from more than one gland to obtain sufficient material.

*Oestrus cycle*

This was determined by daily vaginal smears taken 3 hr after the beginning of the light period. This was continued for 1–2 weeks before the rats were used.

*Extraction of DNA*

The mammary gland was weighed, minced with scissors and then disrupted with a Silverson (Silverson Limited, London) homogeniser in 5 ml water. Ten ml of N perchloric acid (PCA) was added and the suspension centrifuged. The pellet was washed three times each with 10 ml of 0·5 N PCA followed by three washings each of 10 ml ethanol: ether: chloroform (2 : 2 : 1).

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*Table* 1. *Incorporation of* [$^3H$] *thymidine into rat mammary gland and uterus*

| | DPM/μg DNA | | DPM/mg weight | | μg DNA/g weight | |
|---|---|---|---|---|---|---|
| | Mammary gland | Uterus | Mammary gland | Uterus | Mammary gland | Uterus |
| Oestrus | 232±86 | 88±13 | 118±25 | 200±31 | 588±45 | 2284±117 |
| Oestrus+DMBA | 35±8 | 45±4 | 13±3 | 122±33 | 667±98 | 2611±569 |
| Oestrus+ benzanthracene | 44±11 | — | 31±11 | — | 753±117 | — |
| Prooestrus | 44±13 | 328±163 | 47±25 | 897±535 | 823±184 | 2446±223 |
| Prooestrus+DMBA | 9±2 | 119±49 | 6±2 | 197±77 | 994±159 | 1615±127 |
| Ovariectomized | 77±25 | — | 101±52 | — | 759±138 | — |
| Ovariectomized+1 μg oestradiol | 95±22 | — | 102±21 | — | 1160±243 | — |
| Ovariectomized+ 3×10 μg oestradiol* | 280±46 | — | 565±110 | — | 2446±1354 | — |
| Ovariectomized+3× 10 μg oestradiol+ DMBA | 50±17 | — | 71±27 | — | 1398±62 | — |

Results are expressed as mean±S.E.M. for at least 4 estimations.
*Only 3 experiments were carried out with this group.

This removed all of the acid-soluble tritium. The insoluble residue was heated for 15 min. at 70° with 1·5 ml 0·5 N PCA and 0·5 aliquots of the soluble fraction taken for DNA [6] and tritium estimations. The tritium was counted in a Packard liquid scintillation spectrometer model 3003 and quenching was assessed by channel ratio [7].

In six experiments, [$^3$H] thymidine labelled mammary glands were removed without fixation in Bouin's fluid and the DNA extracted as above. The specific activity of the DNA (disintegrations/min/μg DNA) was the same as in the fixed samples, indicating that fixation does not alter this parameter.

## RESULTS

*Effect of oestrous cycle and hydrocarbons on thymidine incorporation*

In the mammary gland, [$^3$H] thymidine incorporation per μg DNA was higher at oestrus than at prooestrus ($p<0·001$) whilst the converse was true of the uterus ($p<0·05$), Table 1. In both tissues, there was a higher mean DNA content at prooestrus than at oestrus but this was not statistically significant ($p>0·05$).

Ovariectomy 7 days prior to [$^3$H] thymidine injection lowered the incorporation in the mammary gland relative to the oestrus level. A single injection of 1 μg oestradiol 24 hr prior to the [$^3$H] thymidine did not affect [$^3$H] thymidine incorporation but three daily injections, each of 10 μg, increased incorporation to the oestrus level.

Administration of DMBA 24 hr prior to the [$^3$H] thymidine markedly inhibited both the prooestrus and oestrus levels of incorporation in mammary gland ($p<0·02$ and $0·001$ respectively). The non-carcinogenic hydrocarbon benzanthracene had a similar effect at oestrus but was not tested at prooestrus. DMBA also inhibited the effect of oestradiol on thymidine incorporation.

*Thymidine incorporation into the terminal buds and central area of the mammary gland*

In six experiments, the incorporation was compared between the central area and terminal buds from the same gland. As the terminal buds contained too little DNA to measure, this comparison was made per g wet weight. Both at prooestrus and oestrus there was a greater incorporation into the terminal buds than into the central region and this difference was greater at prooestrus than at oestrus. The ratio of incorporation into the terminal buds: central area of the same gland was 10 : 2 at prooestrus and 3 : 2 at oestrus. DMBA inhibited [$^3$H] thymidine incorporation into the terminal buds.

All of the above analyses were carried out

on mammary tissue from gland 3. Incorporation into the central areas of the other glands occurred to about the same extent except that there was a tendency for a lower incorporation into gland 6.

## DISCUSSION

The observation that maximal [$^3$H] thymidine incorporation occurred at oestrus in the mammary gland and at prooestrus in the uterus indicates that these two tissues have different sensitivities to endogenous hormones.

Hydrocarbon inhibition of DNA synthesis has been reported for a number of tissues both *in vivo* and *in vitro* [3–5] but the mechanism is unknown. In the present experiments, it is possible that the hydrocarbon is affecting DNA synthesis indirectly via the endocrine glands. The lack of effect on RNA synthesis [8] would indicate that it is not simply a general toxic effect of the hydrocarbon. Since inhibition occurs in tissues in which the hydrocarbon is not carcinogenic and, in the present experiments, the non-carcinogenic hydrocarbon, benzanthracene, was also inhibitory, it is unlikely that it is directly related to carcinogenesis. However, it may contribute to some, as yet undefined, carcinogenic effect of the hydrocarbon.

Although the elevation of DNA in the DMBA treated animals was not statistically significant it is consistent with the report of Alfred [8] that DMBA increased cellular DNA, and also suggests that this hydrocarbon may affect DNA breakdown.

## SUMMARY

*The* in vivo *incorporation of* [$^3$*H*] *thymidine into rat mammary gland has been studied under different conditions. Incorporation into DNA was higher in glands obtained at oestrus than at prooestrus and decreased after ovariectomy. The effect of ovariectomy was prevented by oestradiol injection. The incorporation of* [$^3$*H*] *thymidine was greater in the terminal buds of the mammary gland than in the central area of the gland.*

*DMBA markedly inhibited incorporation into DNA of glands obtained at both oestrus and prooestrus. It also inhibited the incorporation into uterine DNA. The non-carcinogenic hydrocarbon, benzanthracene, also inhibited DNA synthesis in mammary gland.*

*It is concluded that the DMBA inhibition of DNA synthesis is not directly related to the carcinogenic action of this hydrocarbon.*

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# Activité Biologique des Différentes Zones Obtenues par Ultracentrifugation du Facteur Lacté de la Souche de Souris PS sur Gradients Préformés de Ficoll

J. MOURIQUAND, C. MOURIQUAND et C. VIALA

*Laboratoire de Biologie Cellulaire, Centre d'Etudes Nucléaires de Grenoble, et Laboratoire d'Histologie, Faculté de Médecine de Grenoble, 38–La Tronche, France*

DES TENTATIVES d'isolement du facteur lacté de Bittner, par ultracentrifugation de lait ou d'extraits acellulaires de tumeurs mammaires sur gradients préformés de Ficoll et de tartrate de potassium, ont été effectuées par différents auteurs [1–4]. Si les particules virales de type B ont pu être obtenues dans une zone bien précise, l'activité biologique est beaucoup plus largement distribuée.

L'ultracentrifugation sur gradients préformés de Ficoll, décrite par Moore [1], a été appliquée à différentes sources de virus de la souche de souris PS: lait, extraits acelullaires de tumeurs mammaires, milieux de cultures de tumeurs mammaires en couche monocellulaire. On sait que cette souche de souris développe spontanément tumeurs mammaires et leucémies [5]. L'isolement des deux agents oncogènes en cause pouvait être espéré par cette méthode.

Dans la première partie de ce travail, l'activité biologique des extraits acellulaires de référence a été estimée en fonction de leur mode de confection, de leur température de conservation et des souches de souris inoculées. Ces résultats préliminaires acquis, les différentes zones obtenues après ultracentrifugation sur gradients de Ficoll ont été testées au point de vue biologique. Des contrôles ultrastructuraux des zones inoculées ont été effectués à trois reprises.

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## MATERIEL ET METHODES

I. *Source de virus*

*A. Le Lait.* Il provient de la souche de souris PS (10 échantillons) et de témoins C3H/eB/Gif (7 échantillons) dont l'incidence tumorale mammaire est inférieure à 2% dans notre élevage.

La collection du lait est effectuée suivant la méthode de McBurney *et al.* [6]. Il est conservé au froid (+4°C ou −60°C). Le délai de stockage ne dépasse jamais 15 jours.

Le lait dégraissé et décaséiné est pelletisé à 21.000 t/min, à 0°C, pendant une heure [1]. Le pellet final est resuspendu dans du tampon Tris (0,01 M tampon Tris pH 7,4+0,14 M NaCl+0,04% de sérum albumine).

*B. Tumeurs mammaires et glandes mammaires en lactation.* Les extraits sont effectués à partir de tumeurs spontanées de la souche de souris PS ou induites par ces mêmes extraits (passage I), ainsi que de glandes mammaires en lactation, en utilisant deux techniques différentes: —

après broyage des tumeurs, dans du tampon Tris (10 ml pour 1 g), à l'Ultraturrax (20.000 t/min × 50 secondes), les plus gros débris sont éliminés par deux centrifugations successives (3.000 t/min pendant 20 min, puis 15 min à 15.000 t/min). Le surnageant est conservé (technique de Gross) [7]—ou bien l'extraction implique l'utilisation de Fréon et consiste en plusieurs centrifugations suivies d'une pelletisation finale (technique de Moore) [8].

*C. Cellules tumorales en culture.* Les tumeurs sont prélevées stérilement, trypsinées selon les méthodes classiques, et mises en culture en couches monocellulaires [9].

Après trois jours de culture, les cellules détachées, broyées à l'Ultraturrax dans leur milieu de culture, sont soumises à des centrifugations différentielles comparables à celles utilisées par Moloney [10].

Le pellet obtenu après la dernière centrifugation de 30.000 t/min est mis en suspension dans 0,5 ml de tampon Tris.

II. *Ultracentrifugation sur gradient de densité*

*A. Gradients de Ficoll.* Les gradients de concentration, préformés de Ficoll, sont préparés par superposition de solutions de densité décroissante (50% à 5% dans du tampon Tris puis laissés 4 jours à +4°C pour permettre la diffusion, selon la technique de Moore [1].

Un demi-millilitre de suspension virale est déposé au sommet du gradient. Après ultracentrifugation des tubes, durant 1 h, à 39.000 t/min (Spinco L, rotor SW 39), les différentes zones observées sont collectées après ponction du fond du tube, par fractions de 5 gouttes, ou bien par capillarité à la surface du gradient.

Après sédimentation des zones à 28.000 t/min pendant 20 min, les pellets obtenus sont resuspendus dans 1,5 ml de tampon et inoculés à des souriceaux de souches diverses.

Le contrôle ultrastructural a été effectué, soit après coloration négative des différentes fractions prélevées sur grilles de Formwar (technique de Brenner et Horne [11]) (Dr. Hollmann) soit après inclusion des pellets fixés à la glutaraldéhyde (Dr. Haguenau).

III. *Tests biologiques*

Différentes souches de souris sont inoculées: C3H/eB/Gif et BALB/c, dont l'incidence tumorale est respectivement de 2 et 0% dans notre élevage, ainsi que des souris de la souche PS.

Les animaux sont injectés par voie intrapéritonéale à raison de 0,1 ml par souris, dans les 24 heures suivant la naissance.

Les témoins sont constitués par des souris de souche:

—BALB/c/Gif: 46 femelles et 17 mâles non-inoculés,

—C3H/eB/Gif: 40 femelles et 20 mâles non-inoculès,

—PS: 49 femelles de la 32e génération, 39 mâles des 21e et 25e générations, observés jusqu'à 15 mois.

Les souris sont placées par cage de 10, sans séparation de sexe, alimentées suivant le régime de Haddow, eau ad libitum.

Tous les animaux sont maintenus en observation jusqu'à l'apparition de tumeurs ou de différents signes d'altération de l'état général. Les souris sont alors sacrifiées. Une autopsie est pratiquée, suivie d'un prélèvement systématique des foie, rate, reins et organes tumoraux.

La fixation est faite au Bouin Hollande, la coloration à l'hématoxyline, à la phloxine, et au safran.

## RESULTATS

I. *Activité biologique des extraits acellulaires de tumeurs mammaires en fonction de leur mode de confection et de conservation*

Quatre cent treize souris de souches C3H/eB, BALB/c et PS ont été inoculées, dans les 24 h suivant la naissance, avec des extraits

*Tableau* 1. *Infectivité sur souris de souche BALB/c d'extraits acellulaires confectionnés au fréon, en fonction de la température de stockage*

| Injections | Nombre d'animaux BALB/c inoculés | Survivent à 6 mois | TM | AM | % |
|---|---|---|---|---|---|
| Immédiate | 44 | 19♀+12 ♂ | 12 | 8 | 63 |
| Après 24 h à +4°C | 40 | 9♀+ 9 ♂ | 4 | 6,8 | 44,5 |
| 1 mois à −60°C | 31 | 9♀+ 6 ♂ | 7 | 7,5 | 77,7 |

TM = tumeurs mammaires.
AM = âge moyen en mois.

*Tableau 2. Effets biologiques d'extraits acellulaires de tumeurs mammaires confectionnés au fréon*

| Souches inoculées | EA de tumeurs mammaires | | | EA de glandes mammaires en lactation | | |
|---|---|---|---|---|---|---|
| | TM | AM | L | AM | TM | AM |
| 21 C3H/eB | 0 | | 0 | | — | — |
| Témoins 46 C3H/eB | 1/46<br>2,1% | 32 | 0 | | | |
| 115 BALB/c | 12/24<br>50% | 7,6 | 0 | | 11/15<br>73% | 7,8 |
| Témoins 42 BALB/c | 0 | | 0 | | | |
| 26 PS ♀ | 6/7 | 8,5 | 1/7 | 5,5 | — | — |
| 26 PS ♂ | 0 | | 3/9 | 8,3 | — | — |
| Témoins PS | | | | | | |
| 43 ♀ | 26/43 | 10,1 | 1 | 11 | — | — |
| 39 ♂ | 0 | | 3/39 | 9 | — | — |

TM = nombre de tumeurs mammaires/nombre de femelles survivant à 6 mois.
AM = âge moyen en mois.
L = nombre de leucémies/nombre d'animaux survivant à 6 mois.
— = non testés.

acellulaires de tumeurs mammaires spontanées ou de glandes mammaires en lactation de la souche de souris PS et de tumeurs mammaires induites par ces mêmes extraits (passage I).

Une importante mortalité est observée chez les animaux de souches C3H/eB et BALB/c; seules les femelles survivant à six mois ont servi à l'établissement des tests biologiques.

Deux modes de préparation ont été utilisés, les techniques de Gross et de Moore. L'activité biologique de tels extraits, en fonction de leur mode de conservation, est étudiée ci-dessous.

*A. Extraits acellulaires confectionnés selon la méthode de Moore.* L'infectivité de l'extrait inoculé, immédiatement après sa confection, est comparée à celle de produits conservés 24 h à +4°C et 1 mois à −60°C. (Les résultats sont consignés au Tableau 1).

Il n'y a pas de perte significative d'infectivité entre l'extrait frais, conservé 24 h à +4°C, et un mois à −60°C ($\chi^2=5{,}476<5{,}999$ pour ddl=2).

Les résultats obtenus, en fonction des souches de souris inoculées, sont donc établis globalement, sans tenir compte du mode de conservation de l'extrait (Tableau 2):

—Soixante-douze souris BALB/c nouveau-nées ont été inoculées avec des extraits acellulaires de tumeurs mammaires, 24 femelles survivent à 6 mois, 12 tumeurs mammaires sont signalées à un âge moyen de 7,6 mois, soit 50%.

—Quarante-trois souris nouveau-nées BALB/c sont injectées avec un extrait acellulaire de glandes mammaires en lactation, prélevées sur une souris porteuse de tumeur mammaire. Onze femelles sur 15 développent une tumeur mammaire à un âge moyen de 7,8 mois, soit 73% de malignité.

—Vingt-six souris nouveau-nées de la souche PS sont inoculées avec des extraits acellulaires de tumeurs mammaires: 100% de malignité sont observés chez les 7 femelles survivant à 6 mois, 6 tumeurs mammaires à un âge moyen de 8,5 mois, et 1 leucémie à 5,5 mois. De tous les mâles inoculés, seuls ceux de la souche PS ont développé des leucémies, soit 3 sur 9 (33%), à un âge moyen de 8,3 mois. Ils sont seuls consignés sur ces tableaux.

Aucune malignité n'a été constatée chez les 8 femelles C3H/eB observées jusqu'à 14 mois.

*B. Extraits acellulaires effectués selon la méthode de Gross.* La comparaison de l'infectivité de ces extraits, en fonction de leur conservation de 24 à 48 h à +4°C, et d'un mois à −60°C et −195°C, est rapportée au Tableau 3.

L'activité biologique des produits maintenus à −60°C est conservée par rapport à ceux inoculés immédiatement après leur confection ($\chi^2=0{,}22$). Mais la perte d'infectivité des extraits conservées de 24 à 48 h à +4°C est significative ($\chi^2=8{,}75$). Le petit nombre des femelles survivant 6 mois à l'inoculation d'extraits conservés à −195°C ne permet pas d'établir de calcul statistique. Il semble cependant que la conservation à trés basse

*Tableau 3. Infectivité des extraits acellulaires de tumeurs mammaires confectionnés selon la méthode de Gross, en fonction de leur température de conservation*

| EA de tumeurs mammaires | Nombre de BALB/c inoculées | Survivent à 6 mois | TM | AM | % |
|---|---|---|---|---|---|
| Injection immédiate | 37 | 15♀ | 13 | 6,4 | 86 |
| +4°C | 36 | 18♀ | 3 | 6 | 16,6 |
| −60°C | 43 | 17♀ | 11 | 6 | 64,7 |
| −195°C | 21 | 3♀ | 3 | 7,3 | 100 |

EA = extraits acellulaires.
TM = tumeurs mammaires.
AM = âge moyen en mois.

température n'affecte que peu l'activité biologique: 3 femelles sur 3 ont développé 1 tumeur mammaire.

L'activité biologique des extraits acellulaires effectués selon la méthode de Gross, en fonction des souches de souris inoculées, est représentée au Tableau 4. Les produits

*Tableau 4. Activité biologique des extraits acellulaires confectionnés selon la méthode de Gross, en fonction des souches inoculées*

| Souches inoculées | Nombre d'animaux | TM | AM | % |
|---|---|---|---|---|
| BALB/c | 91 | 27/35 | 6,6 | 77 |
| C3H/eB | 110 | 1/45 | 10 | 2,2 |

TM = tumeurs mammaires/nombre de femelles survivant à 6 mois.
AM = âge moyen en mois.

conservés à +4°C sont exclus de cette estimation:

—Cent dix souris nouveau-nées C3H/eB, sans facteur lacté, ont été injectées; 45 femelles survivant à 6 mois; une tumeur mammaire est observée à 10 mois.

Quatre-vingt-onze souris BALB/c sont inoculés, 35 femelles survivent à 6 mois, 27 développent des tumeurs mammaires à un âge moyen de 6,6 mois. Les mâles n'ayant présenté aucune malignité, sont exclus des tableaux.

La comparaison des résultats obtenus avec les extraits acellulaires de tumeurs mammaires, confectionnés selon les techniques de Gross et de Moore, est établie au Tableau 5.

On observe respectivement 77 et 50% de tumeurs mammaires chez les souris de souche BALB/c. Cette différence n'est pas significative $=\chi^2=1{,}909, <3{,}841$:

*Tableau 5. Comparaison des résultats obtenues avec les méthodes de Gross et de Moore*

| Souches inoculées | EA Technique Gross | EA Technique Moore |
|---|---|---|
| C3H/eB | 2,2% TM AM=10 | 0 |
| BALB/c | 77% TM AM=6,6 | 50% TM AM=7,6 |

EA = extraits acellulaires.
TM = tumeurs mammaires.
AM = âge moyen en mois.

## II. *Activité biologique des zones de gradients obtenues après ultracentrifugation sur ficoll*

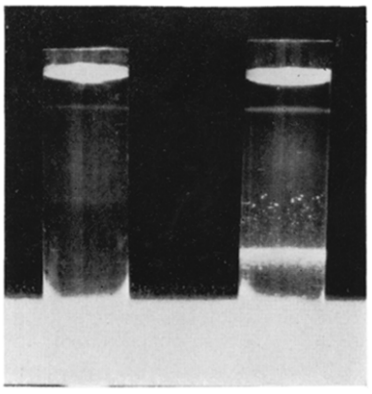
L'activité biologique des zones obtenues après ultracentrifugation sur gradients préformés de Ficoll a été recherchée en utilisant différents produits biologiques susceptibles de contenir le facteur lacté de la souche de souris PS. Ces investigations ont concerné 10 échantillons de lait de la souche PS, 3 tumeurs mammaires en cultures monocellulaires, au 3e jour de culture, et 4 extraits acellulaires de tumeurs mammaires confectionnés selon la méthode de Moore.

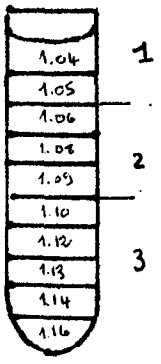
Les échantillons de lait de la souche PS ont permis d'observer après ultracentrifugation sur gradients de Ficoll une succession de zones opalescentes dans des régions de densité s'élevant de 1,04 à 1,09 (Fig. 1).

Sept échantillons témoins de lait de la souche C3H/eB/Gif ont montré, après ultracentrifugation, deux zones opalescentes à la partie supérieure du tube et une zone granuleuse de densité plus élevée.

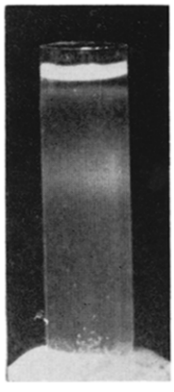
A partir des échantillons de tumeurs mammaires en culture, les zones obtenues sont comparables à celles observées après ultracentrifugation de lait de la souche PS.

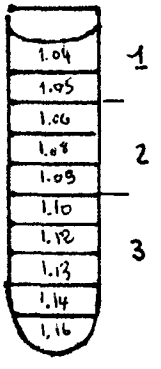
DENSITES



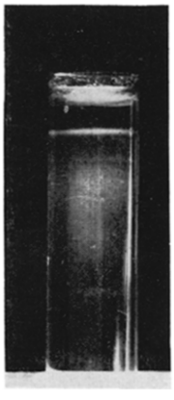


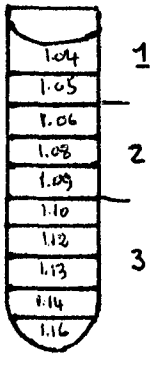

LAIT : PS C3H/eB/Gif






TUMEURS MAMMAIRES EN CULTURE






TUMEURS MAMMAIRES PS

*Fig.* 1. *Zones observées après ultracentrifugation du lait, de tumeurs mammaires et de leurs milieux de culture.*

(to face p. 118)

*Tableau* 6. *Activité biologique des zones obtenues après ultracentrifugation de lait sur gradients préformés de Ficoll*

| Zones inoculées | Souches | Survivent à 6 mois | TM | Tumeurs diverses |
|---|---|---|---|---|
| Zone 2 | 25 C3H/eB | 10♀ 10♂ | 4<br>AM=14 | 1<br>AM=15 |
| Zone 3 | 34 C3H/eB | 7♀ 8♂ | 5<br>AM=17,3 | |

TM = tumeurs mammaires.
AM = âge moyen en mois.

Les extraits de tumeurs spontanées de la souche PS fournissent des images légèrement différentes: deux premières zones analogues à celles obtenues avec le lait et les milieux de cultures, et une zone de densité 1,14.

Pour effecteur les tests biologiques, les tubes sont divisés arbitrairement en trois fractions (Fig. 1) qui servent aux inoculations, sans dilution préalable.

1. *Infectivité des zones après ultracentrifugation de lait sur gradients préformés de Ficoll* (Tableau 6)

Seules les zones 2 et 3 ont été inoculées à 59 souriceaux C3H/eB. Quarante et 71% de tumeurs mammaires sont apparues à un âge moyen de 14 et 17,3 mois, respectivement; 1 léiomyosarcome utérin est signalé à 1,5 mois.

2. *Infectivité des zones après ultracentrifugation de milieux de culture de tumeurs mammaires* (Tableau 7)

Les zones 1 et 2 ont été injectées à 21 souriceaux C3H/eB et BALB/c. Le nombre de femelles survivant à 6 mois est faible. Le pourcentage de tumeurs mammaires élevé concerne toutes les femelles BALB/c, 2 sur 2 (zone 2), toutes les femelles C3H/eB, 4 sur 4 (zone 1) et 3 sur 4 (zone 2). Les âges d'apparition sont tardifs, âge moyen 18,5 et 19 mois pour les souris de la souche C3H/eB, 11 mois pour les souris BALB/c.

*Tableau* 7. *Activité biologique des zones obtenues après ultracentrifugation de milieux de cultures de tumeurs mammaires sur gradients préformés de Ficoll*

| Zones inoculées | Souches | Survivent à 6 mois | TM |
|---|---|---|---|
| Zone 1 | 4 C3H/eB | 4♀ | 4/4<br>AM=19 |
| Zone 2 | 8 C3H/eB | 4♀ | 3/4<br>AM=18,5 |
| | 9 BALB/c | 2♀ 3♂ | 2/2<br>AM=11 |

TM = tumeurs mammaires.
AM = âge moyen en mois.

3. *Infectivité des zones après ultracentrifugation d'extraits acellulaires selon la méthode au fréon* (Tableau 8)

Les trois zones ont été inoculées à 67 souris de souches C3H/eB, BALB/c et PS. Une

*Tableau* 8. *Activité biologique des zones obtenues après ultracentrifugation d'extraits acellulaires de tumeurs mammaires sur gradients préformés de Ficoll*

| Zones inoculées | Souches | Survivent à 6 mois | TM | AM | L |
|---|---|---|---|---|---|
| Zone 1 | 9 BALB/c | 3♀ 3♂ | 2/3 | 8,7 | 1 |
| | 2 PS | 2♀ | 1/2 | 7,5 | 1,5 mois |
| | 14 C3H/eB | 3♀ 2♂ | 0/3 | | |
| Zone 2 | 7 BALB/c | 6♀ 1♂ | 4/6 | 6,8 | |
| | 2 PS | 1♀ 2♂ | 1/1 | 8,5 | |
| | 14 C3H/eB | 14♂ | | | |
| Zone 3 | 11 BALB/c | 2♀ 1♂ | 0/2 | | 1 |
| | 3 PS | 2♀ 1♂ | 1/2 | 8 | 16 mois |
| | 8 C3H/eB | 3♀ 1♂ | 1/3 | 14 | |

TM = tumeurs mammaires.
AM = âge moyen en mois.
L = leucémies.

*Tableau* 9. *Activité biologique des zones obtenues après ultracentrifugation du facteur lacté de la souche PS sur gradients préformés de Ficoll*

| Zones inoculées | Souches inoculées | | |
|---|---|---|---|
| | C3H/eB ♀ | BALB/c ♀ | PS ♀ |
| Zone 1 | 4 TM/7<br>AM=19 | 2 TM/3<br>AM=8,7 | 1 TM<br>1 L /2 |
| Zone 2 | 7 TM/14<br>AM=15,1<br>1 tumeur diverse à 3,5 mois | 6 TM/8<br>AM=7,3 | 1 TM/1<br>AM=8,5 |
| Zone 3 | 6 TM/10<br>AM=15,8 | 1 L/2 | 1 TM/2<br>AM=8 |

TM = tumeurs mammaires.
AM = âge moyen en mois.
L = leucémies.

seule tumeur mammaire à 14 mois est observée chez les souris de la souche C3H/eB après inoculation de la zone 3. Pour les souris BALB/c, 2 tumeurs mammaires sur 3 après inoculation de la zone 1,4 sur 6 avec la zone 2, et 1 leucémie avec la zone 3, sont observées. En ce qui concerne les femelles de la souche PS, une tumeur mammaire et 1 leucémie très précoce sont observées avec la zone 1; 2 tumeurs mammaires avec les zones 2 et 3.

Le Tableau 9 réunit globalement les malignités induits par les zones de gradients en fonction des souches inoculées, réunissant ainsi les résultats obtenus avec le lait, les tumeurs mammaires en culture et les extraits acellulaires de tumeurs mammaires. Les observations ainsi réunies sont plus représentatives, car elles conernent un plus grand nombre de femelles survivantes à 6 mois. On voit que l'activité biologique est étalée sur toute la hauteur du gradient.

4. *Les contrôles ultrastructuraux des zones de gradients.* Un contrôle ultrastructural après coloration négative sur grilles a été effectué sur chacune des zones obtenues après ultracentrifugation de lait (Dr. Hollmann). Des particules virales ont été observées dans la seule zone 2.

A une autre reprise, les zones 2 et 3, obtenues après ultracentrifugation d'extraits acellulaires de tumeurs mammaires, ont été soumises à un contrôle ultrastructural qui y a révélé la présence de particules B peu nombreuses (Dr. Haguenau).

## DISCUSSION

Avant d'entreprendre l'étude de l'activité biologique des différentes fractions obtenues aprés ultracentrifugation d'extraits acellulaires de tumeurs mammaires de la souche de souris PS sur gradients préformés de Ficoll, il était indispensable de connaître l'infectivité de ces extraits en fonction de leur mode de confection et de leur température de conservation.

L'analyse des différentes modes d'isolement du facteur lacté à partir du lait ou de tumeurs mammaires utilisés par les travaux contemporains montre une certaine similitude. Les techniques comportent une ou plusieurs centrifugations à vitesse lente, l'adjonction de versène ou d'alphachemotrypsine pour dissocier les agrégats de protéines, et une ou plusieurs centrifugations à grande vitesse. Il existe de grandes variations dans le choix de la vitesse et de la durée de l'ultracentrifugation en fonction des auteurs, allant de 23.000 *g*/60 min [12] à 198.000 *g*/12 min [4]. Moore a remplacé, pour la confection des extraits acellulaires de tumeurs mammaires, le versène par un mode de purification au fluorocarbone, suivi d'une ultracentrifugation à 39.000 t/min, pendant 1 h. C'est cette technique qui a été appliquée ici. Il peut paraître surprenant d'avoir également adopté la technique d'extraits utilisée par Gross pour l'obtention de son passage leucémigène activé. La souche de souris PS développant spontanément tumeurs mammaires et leucémies, il semblait nécessaire d'utiliser un mode de confection d'extraits qui respecterait un éventuel virus leucémigène présent dans les tumeurs mammaires de la souche.

Pour ces 2 différents types d'extraction, il était indispensable d'établir si le mode de conservation des extraits à basse température affectait leur activité biologique.

Si Moore [13] signale que la congélation suivie de décongélation altère la morphologie des particules B sans affecter l'activité biologique, Graff [14] observe que le virus est stable au froid pendant de longues périodes, mais dénaturé par congélation, élévation thermique etc. Enfin, Dmochowski [15] remarque que le facteur lacté présent dans les tissus tumoraux n'est pas altéré par un séjour prolongé à −79°C, mais que l'infectivité d'extraits acellulaires conservés dans les mêmes conditions reste à déterminer.

Il n'y a pas de différences statistiquement significatives entre l'infectivité des extraits effectués selon la méthode de Gross, inoculés immédiatement après leur préparation ou conservés 1 mois à −60°C et −195°C: 77% de tumeurs mammaires à un âge moyen de 6,4 mois chez les souris de souche BALB/c. Un séjour de 24 à 48 heures à +4°C altère l'activité biologique. On peut se demander si les lysosomes, demeurés intacts dans la préparation, ne libèrent pas leurs enzymes au cours du séjour à +4°C, entraînant ainsi une dégradation du virus.

La technique de purification au fréon, plus élaborée, conserve l'action biologique des extraits, quelle que soit leur température de conservation.

Dans les conditions expérimentales adoptées, les 2 modes d'extraction sont comparables, mais il apparaît certain que seule l'étude de l'infectivité de différentes dilutions aurait fourni des éléments de certitude.

Dans la 2e partie de ce travail, l'activité biologique des différentes zones obtenues après ultracentrifugation sur gradients de Ficoll est établie en fonction des souches inoculées. Elle s'étale sur toute la hauteur du gradient.

De nombreux travaux récents ont utilisés la technique d'ultracentrifugation sur gradients pour l'isolement du facteur lacté. Certains, comme Sykes [2], n'ont effectué que des contrôles ultrastructuraux. Hageman n'a pratiqué de tests biologiques qu'avec la seule zone contenant des particules B visibles au microscope électronique [4–16]. Enfin, Nowinski *et al.* [17] ont utilisé cette technique pour des tests immunologiques, les contrôles ultrastructuraux effectués sur les différentes zones obtenues ont objectivé des virions intacts dans 2 zones sur 3 et dans le pellet obtenu.

Dans des conditions expérimentales comparables aux nôtres, le contrôle de l'activité biologique et de l'aspect ultrastructural des différentes zones obtenues après ultracentrifugation de lait sur gradients préformés de Ficoll a été effectué par Moore [1, 18] et Hall [3]. Il signalent tous deux l'étalement de l'activité biologique sur toute la hauteur du gradient.

Hall, étudiant des laits de souches diverses avec et sans facteur lacté, observe pour la souche C3H/An, à haute incidence tumorale, des particules virales de 70 et 100 mμ, dans toutes les zones du gradient, avec un maximum de concentration dans une zone précise qui est celle où l'activité biologique se maintient aux dilutions les plus élevées ($10^{-5}$).

La corrélation entre la présence de particules virales et l'infectivité n'est pas stricte, certaines zones de lait, riches en particules, sont biologiquement inactives. Il pourrait s'agir du "nodule inducing virus" de Pitelka *et al.* [19], qui est morphologiquement identique au facteur lacté. En outre, comme dans le présent travail, pour le lait des souris de souches BALB/cf C3H, le petit nombre de particules virales observées ne correspond pas à l'activité biologique.

Moore, à propos du lait de la souche de souris RIII, signale un maximum d'infectivité allant jusqu'à des dilutions de $10^{-6}$ pour la zone où s'observent les particules B intactes; les autres zones sont infectantes à des dilutions moindres, sans qu'il y ait de correspondance avec des structures virales conservées.

Miroff, après ultracentrifugation du lait sur gradients de CsCl, constate que l'activité biologique ne correspond pas aux zones contenant des particules virales de type B [20]. Il fait les mêmes remarques à propos du lait prélevé dans l'estomac des souriceaux où l'infectivité se trouve dans le surnageant alors que les particules virales sont dans le culot [21].

Il est possible que, dans la présente étude, l'intégrité des particules B ne soit pas conservée au cours de la confection des extraits acellulaires de tumeurs mammaires, d'autant que ceux-ci sont effectués à partir d'organes conservés à −60°C, pendant quelques jours. Moore [13] et Hageman [4] signalent des altérations morphologiques des particules virales par la congélation, sans atteinte de l'infectivité qui peut être le fait du nucléoïde libéré [3]. Cet argument ne s'applique pas aux milieux de cultures de tumeurs mammaires qui sont utilisés sans congélation préalable. Si la particule B, décrite par Bernhard [22], est en jeu dans la transmission des tumeurs mammaires murines, il est admissible qu'elle soit extrêmement vulnérable, en particulier aux différentes méthodes de purification auxquelles elle est soumise, et qu'elle libère alors des sous-unités, elles-mêmes infectantes.

La diffusion de l'activité biologique sur toute

*Tableau* 10. *Activité biologique du facteur lacté, en fonction des vitesses de sédimentation utilisées par différents auteurs*

| Auteurs | Source de Virus | Vitesse de sédimentation | Infectivité | |
|---|---|---|---|---|
| | | | Pellet | Surnageant |
| Bittner [23] | lait | 110.000 *g*/1 h | + | + |
| Barnum [12] | EA de glandes mammaires | 23.000 *g*/1 h | + | 0 |
| Graff [14] | lait | 120.000 *g*/1 h | + | + |
| Passey [28] | EA de tumeurs mammaires | 60.000 *g*/2 h | + | + |
| Moore [18] | lait (gradient) | 39.000 tm/1 h | + | + |
| Miroff [20] | lait | 125.000 *g*/75 min | 60% | 50% |
| Miroff [21] | lait (estomac) | 100.000 *g*/1 h | 0 | + |
| | | | Paticules B+ | + |
| Hall [3] | lait | 22.500 tm/1 h | + | + |
| | gradient | 37.500 tm/5 h | + | + |

EA = Extraits acellulaires.

la hauteur du gradient est en accord avec diverses tentatives de sédimentation du facteur lacté, présentées au Tableau 10.

Il peut paraître surprenant que le facteur lacté soit sédimenté par une centrifugation de 23.000 *g* pendant 1 heure [12], alors qu'il ne l'est pas par une centrifugation de 110.000 *g* pendant le même temps [23]. Des travaux récents d'O'Connor [24] sur l'effet de centrifugations différentielles sur le virus du sarcome de Moloney (M.S.V.) éclairent singulièrement notre propos. Après une centrifugation, à 5.000 t/min, d'extraits tumoraux, le pellet contient 50% de virus compétent. Le surnageant est porteur de virus défectif. La centrifugation du surnageant, à 22.500 t/min, pendant 30 min, fait apparaître 40% de M.S.V. compétent. La présence de virus compétent dans le culot de centrifugation est affectée par la qualité du milieu utilisé: favorisée par un milieu à base de P.B.S., inhibée par une solution contenant 0,306 M de citrate de potassium.

Ces très intéressantes observations mettent l'accent sur les effets insoupçonnés de centrifugations à basses vitesses sur la sédimentation d'un autre virus murin.

Les leucémies observées chez les animaux inoculés méritent de retenir l'attention. Pour les souris de la souche PS, ce sont essentiellement les mâles qui ont développé ce type de malignité (3 sur 9, à un âge moyen de 8,3 mois), et une femelle à 6 semaines. Si l'activation d'un virus latent, propre à cette souche, peut être mis en cause, ce n'est pas le cas pour la souris femelle de souche BALB/c ayant développé une leucémie à l'âge de 16 mois, après inoculation de la zone 3, obtenue après ultracentrifugation d'extrait acellulaire de tumeur mammaire sur gradient de Ficoll. La transmission du virus leucémigène, par le lait, signalé à diverses reprises [25–27] explique vraisemblablement ces résultats. Les tests biologiques, effectués dans des conditions analogues par Hall, Moore et Miroff, n'ont pas induit de leucémie, bien que Hall signale la présence de virus ressemblant à des particules virales de type C, au fond du gradient obtenu après ultracentrifugation du lait de la souche C3H f B.


## RESUME

*L'activité biologique des extraits acellulaires de tumeurs mammaires est appréciée en fonction de leur mode de confection: techniques de Gross et de Moore, après extraction au fluorocarbone. Il n'y a pas de différence significative entre ces deux méthodes. Il n'y a pas de perte d'infectivité après conservation à* −60°C *et* −195°C*; seuls les extraits confectionnés selon la méthode de Gross, et conservés* 24 *à* 48 *h à* +4°C*, ont une perte significative d'infectivité.*

*Ces mêmes extraits, ainsi que des échantillons de lait et de milieux de culture de tumeurs*

*mammaires, sont ultracentrifugés sur gradients préformés de Ficoll. L'infectivité des zônes ainsi obtenues est appréciée sur des souriceaux de souches BALB/c, C3H/eB et PS. Elle s'étale sur toute la hauteur du gradient. Des leucémies sont observées chez les mâles PS et chez une femelle BALB/c.*

*Ces résultats sont discutés en fonction d'études analogues faites par d'autres auteurs.*

## SUMMARY

*Cell free extracts of mammary tumours of the PS strain of mice were performed following Gross's technique and the extraction with fluorocarbon described by Moore. Bioactivity does not display a significative difference with the two methods. There is no loss of infectivity after storage at −60°C and −195°C. A significative loss of infectivity is only observed with cell free extracts performed following Gross's method and kept 24 to 48 hr at 4°C.*

*These extracts, samples of milk and of tissue culture media of mammary tumours, were ultracentrifugated on preformed density gradients of Ficoll. Bioassays with the zones thus obtained were performed on newborn mice of different strains of mice: BALB/c, C3H/eB, PS. Infectivity is spread all over the gradient. Leukemias were observed in PS males and in one female of the strain BALB/c.*

*These results are compared with similar studies of different authors.*

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# The Effect of Cytotoxic Agents on Drug Metabolism

M. G. DONELLI, G. FRANCHI* and R. ROSSO
*Istituto di Ricerche Farmacologiche "Mario Negri" Via Eritrea, 62—20157 Milano, Italy*

VERY little is known about the interaction of antitumoral agents with other drugs. A recent report of Tardiff [1] prompted us to summarize our experiments concerning the effect of cytotoxic drugs on the metabolic activity of liver microsomal enzymes. This investigation is related also to previous studies from this laboratory [2–4] which showed that the drug metabolizing activity of the liver microsomal enzymes in tumor-bearing animals is markedly reduced when compared to controls. Male Sprague-Dawley rats of the average weight of 150±10 g were administered intraperitoneally with a single dose of different cytotoxic compounds, cyclophosphamide, nitrogen mustard ($HN_2$), daunomycin, 5-fluorouracil, methotrexate and hydrocortisone. The animals were sacrificed and the livers were homogenized with 4 volumes of ice-cold 1·15% KCl solution in a Potter type Teflon-homogenizer. The homogenates were centrifuged at 9000 **g** for 20 min; 3 ml of 9000 **g** supernatant, equivalent to 600 mg of liver were mixed with 2 ml of a solution containing NADP (1·5 μM), glucose-6-phosphate (50 μM) magnesium chloride (25 μM), nicotinamide (100 μM), potassium phosphate buffer (280 μM, pH 7·4) and the substrate to be metabo-

*Table* 1. *Impairment of drug metabolism 7 days after a single i.p. administration of some cytotoxic compounds in normal rats* (9000 **g** *liver fraction*). *Controls*=100

| Treatment | Dose mg/kg | mμM metabolite produced | | | Liver proteins |
|---|---|---|---|---|---|
| | | *p*-$NO_2$-phenol | *p*-$NH_2$-phenol | 4-$NH_2$-antipyrine | |
| None | — | 100 | 100 | 100 | 100 |
| Cyclophosphamide | 100 | 56* | 50* | 44* | 88† |
| Nitrogen mustard | 1 | 58* | 56* | 53* | 91 |
| 6-Mercaptopurine | 300 | 82† | 102 | 80 | 96 |
| Hydrocortisone | 100 | 95 | 106 | 88 | 89 |
| Methotrexate | 4 | 80* | 76* | 42* | 86† |
| 5-Fluorouracil | 120 | 55* | 42* | 36* | 100 |
| Daunomycin | 10 | 68* | 57* | 39* | 94 |

*$p<0·01$.
†$p<0·05$.
In control rats the metabolites produced were respectively 215±9 mμM of *p*-$NO_2$ phenol, 294±17 mμM of *p*-$NH_2$ phenol and 187±25 mμM of 4-$NH_2$-antipyrine/100 mg proteins and the protein content was 140±3 mg/g liver.
Each figure is the average of 6 determinations.

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*Fellow, Lions Club Milano Host.

lized (3 μM of para-nitro-anisol or 5 μM of aniline or 5 μM of amidopyrine). The mixture was then incubated for 30 min at 37°C. O-demethylation of para-nitroanisol, para-hydroxilation of aniline, N-demethylation of amidopyrine were determined by measuring respectively the formation of para-nitrophenol, para-amino-phenol and 4-amino-antipyrine according to the method of Gilbert and Goldberg [5]. Protein determinations were performed according to Lowry [6].

Table 1 shows the results obtained 7 days after the treatment. It is evident that all compounds tested, with the exception of hydrocortisone and 6-mercaptopurine, significantly reduced the enzymatic activity of the liver microsomal system even when the protein content of the liver was not significantly affected. Fourteen days after the treatment (results, reported in Table 2) only cyclophosphamide among the tested drugs, keeps the inhibiting effect. These findings are in substantial agreement to those reported by Tardiff and they may be relevant also for clinical conditions, where tumour bearing subjects are usually submitted to a variety of drug combination [7].

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*Table 2. Impairment of drug metabolism 14 days after a single i.p. administration of some cytotoxic compounds in normal rats (9000 g liver fraction). Controls=100*

| Treatment | Dose mg/kg | mμM metabolite produced | | | Liver proteins |
|---|---|---|---|---|---|
| | | *p*-$NO_2$-phenol | *p*-$NH_2$-phenol | 4-$NH_2$-antipyrine | |
| None | — | 100 | 100 | 100 | 100 |
| Cyclophosphamide | 100 | 65* | 73* | 45* | 99 |
| Nitrogen mustard | 1 | 89 | 99 | 86 | 89 |
| 6-Mercaptopurine | 300 | 81 | 92 | 108 | 97 |
| Hydrocortisone | 100 | 102 | 109 | 118 | 93 |
| Methotrexate | 4 | 105 | 120 | 85 | 102 |
| 5-Fluorouracil | 120 | 94 | 91 | 96 | 103 |
| Daunomycin | 10 | 106 | 108 | 89 | 102 |

*$p<0.01$.

In control rats the metabolites produced were respectively 174±7 mμM of *p*-$NO_2$-phenol, 243±13 mμM of *p*-$NH_2$-phenol and 140±9 mμM of 4-$NH_2$-antipyrine/100 mg proteins and the protein content was 157±4 mg/g liver.
Each figure is the average of 6 determinations.

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# Positively Charged Amino Groups on the Surface of Normal and Cancer Cells

J. N. MEHRISHI

*Department of Radiotherapeutics, University of Cambridge, Addenbrooke's Hospital, Trumpington Street, Cambridge, England*

## INTRODUCTION

CHANGES in the cell surface may be an important aspect of the differences between normal and cancer cells. Many of the distinctive properties of neoplasms, such as local invasion, metastasis and loss of contact inhibition are believed to depend on changes in the tumour cell membrane and appear to be governed by the magnitude of the surface charge of the cells at least in some systems [1–4]. The role of surface charge of normal cells in connection with adhesion studies [3] and that of tumour cells in connection with the invasiveness [5], lack of contact inhibition and decreased mutual adhesiveness [6, 7] has been the subject of considerable interest as reviewed in references 1 and 2. The net surface charge on tumour cells is negative and the charge density (electron charges per unit area) is high and a decrease in mutual adhesiveness of the tumour cell membrane probably emphasizes a change in its chemical constitution following malignant transformation. It is generally agreed that the adhesive material on the cell surface contains protein. Antibody-like protein on the surface of Ehrlich ascites tumour (EAT) cell has been detected [8, 9; cf. 2]. It is important to define the properties of the cell surface and examine what properties and what type of material transform such that the invasiveness of the tumour cell results. To examine any specific change in the ultrastructure of the tumour cell membrane a detailed examination both on normal and tumour cell surface is evidently necessary. Various approaches have been made to analyse the cell surface material and the cellular ultrastructure, particularly, with the use of cell electrophoresis. So far a great deal of work has been concerned with studying the role of neuraminidase-susceptible N-acetylneuraminic acid (NANA) on the cell surface. NANA plays a dominant role in the biosynthesis of several blood group substances and myxovirus receptors of human and animal origin. NANA is involved in the human M and N specificity and in two other genetic systems also and there are many ways in which NANA may contribute to the development of antigenic structures under direct genetic control (e.g. see ref. 10). Besides being involved in a genetic system, NANA has the general properties of contributing to the bulk of the negative charge of the animal cells [11–14] and of masking antigens, for example tumour antigens.

No data have been available on the positively charged amino groups on the cell surface, clearly identified, presumably because of the lack of suitable mild reagents needed to block groups of high pK such as amino groups. The presence of positively charged groups of high pK greater than 9, such as amino, on the surface of EAT cells [12, 15], human blood platelets [16, 17] and lymphocytes [18, 19] has been suggested. Since protein biosynthesis is under direct genetic control and the gene DNA carries the information necessary to code for a given primary amino acid sequence, the knowledge about these positively charged amino groups on the cell surface could be of importance. Any changes in the amino acid

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sequence of the cell surface proteins/peptides and any intrinsic differences between the surface of different cells could give valuable information about the genetic material controlling the synthesis of the cell surface proteins etc. There is evidence also for the specific role of free amino groups, especially of free $\varepsilon$—$NH_2$ of lysine residues in the blood group activity of M and N blood group substances [20].

The present studies were undertaken to identify the cationogenic (positive) groups believed to be associated with proteins on the surface of EAT cells, human blood platelets and lymphocytes and to determine their relative contribution to the net electrokinetic charge which may have some bearing on the adhesion and aggregation phenomena. If the cationogenic groups can be blocked reversibly by mild chemical treatments to modify the electrokinetic behaviour of cells, reliable estimates of their contribution to the total surface charge can be made. For example, if the amino groups on the cell surface are blocked, the modified cells would exhibit a much higher anodic electrophoretic mobility than untreated cells, the difference arising because of the elimination of the positively charged amino groups from the electrophoretic plane of shear. Data on the reversible blocking of amino groups on the cell surface (EAT, platelets and lymphocytes) carried out by mild chemical reagents are totally lacking. It is important to emphasize the use of reagents which do not lyse the cells or alter the electrokinetic properties irreversibly, because the adsorption of the released intracellular products on the cell surface or any drastic conformational changes would alter the electrokinetic makeup of the cell and meaningful results will not be obtained.

The use of lower aldehydes in blocking the cationogenic groups and modification of the electrokinetic behaviour of cells has been discussed [21]. Aldehyde-treated EAT cells [12] and aldehyde-treated platelets [17] exhibit an anodic mobility 15–21% higher than the untreated cells, presumably because of the elimination of the potential $-NH_3^+$ groups on the cell surface. However, the reactions of aldehydes and cellular proteins are slow and the system is required to stand for 20 days prior to the electrophoretic examination of the cells. During this long period the pH tends to fall and it is necessary to maintain the pH at 7·0–7·4 as pointed out by these authors [21]. French and Edsall [22] many years ago had already pointed out the lack of specificity also as a limitation on the use of aldehydes for blocking the amino groups: formaldehyde and acetaldehyde react with amino, imino, guanidino, hydroxyl and thiol groups.

Recently, Dixon and Perham [23] reported the usefulness of 2-methylmaleic anhydride (citraconic anhydride, CA) and 2,3-dimethylmaleic anhydride (DMA) as reversible blocking agents for protein amino groups in insulin and arginine. The blocking of the cell surface amino groups following reaction with these reagents (CA and DMA) resulting in the introduction of a structure bearing ionizable carboxyl groups into the peripheral regions of cells and the reversal of this process, i.e. the removal of the adduct by lowering the pH to 5 or 6 may be carried out under suitably mild conditions: a positive charge of the amino group is replaced by a negative charge as shown in Fig. 1.

Maleic and citraconic acids in small concentrations had been shown not to have toxic effects on chick fibroblasts in 24 hr culture experiments reported by Friedmann, Marrian and Simon-Reuss [24, 25]. With 3 μM citraconic acid no mitotic inhibition was observed and microscopically, a few clumped metaphases were to be seen but no other abnormalities [25]. It was therefore considered worthwhile to test if CA and DMA could be used to block reversibly the amino groups on the surface of EAT cells, lymphocytes and platelets to modify their electrokinetic behaviour. Electrokinetic studies on cells treated with CA and DMA at pH 6·4 to 6·9 at room temperature (ca. 18°C) were carried out. This range of pH lies in the plateau region of the pH-mobility plots for saline washed cells [12, 16–19] and the results obtained under the most favourable conditions are now available for studying the mosaic distribution of the different types of ionizable groups on the surface of normal and cancer cells. N-ethylmaleimide (NEM) has also been suggested to react with amino groups [26, 27]; electrophoretic mobilities of EAT cells treated with NEM were determined during the past three years and are included in this report.

## MATERIAL AND METHODS

Citraconic anhydride (CA) (British Drug Houses Ltd., Poole, Dorset, U.K.), 2,3-dimethylmaleic anhydride (DMA) (Fluka A.-G., Buchs, Switzerland), N-ethylmaleimide (NEM) (Sigma, London) and other reagents of analytical grade were used. Since the two anhydrides are immiscible with water, solutions of CA and DMA ($10^{-5}$ to $10^{-6}$ M) were

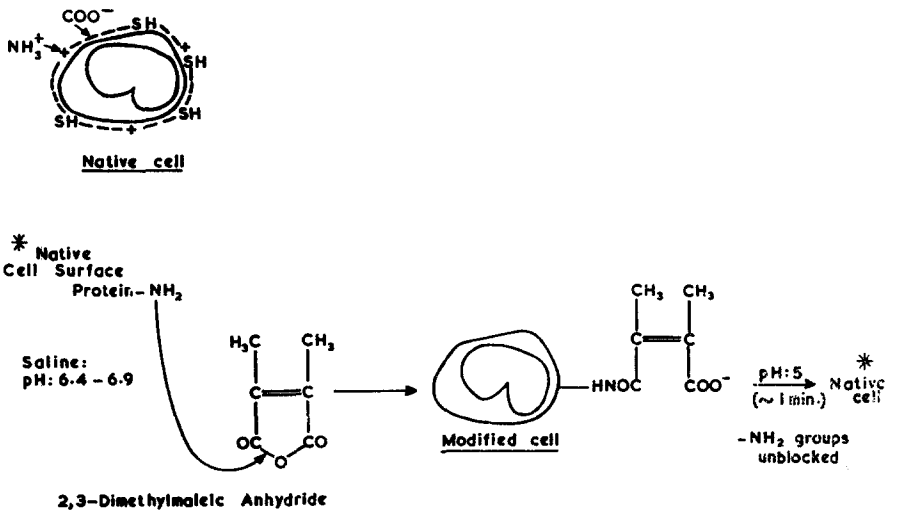


*Fig.* 1. *Reversible blocking of the cell surface amino groups by* 2,3-*dimethylmaleic anhydride* (*or citraconic anhydride*). *Suspending medium saline: pH:* 6·5 *to* 6·9. *Lowering the pH of the suspending medium to* 6 *or* 5 *removes the adduct formed* (*see text for explanations*).

prepared in spectroscopically pure ethanol (British Drug Houses Ltd.). Fresh solutions were prepared immediately before use. Water distilled from Pyrex glass distillation units was used to prepare solutions [28].

Two to three months old Tuck No. 1 male mice bearing Ehrlich ascites carcinoma were killed 6–8 days after transplantation. The cells were harvested and washed twice in saline (0·145 M NaCl, pH adjusted to 7·2±0·2 with the addition of 0·5 N $NaHCO_3$), centrifugation at 60 **g** for 5 min following each wash [29]. Venous blood from healthy individuals was used to separate lymphocytes from defibrinated blood [30] without the use of gelatin sedimentation method as used for other electrokinetic studies [18, 19], and blood platelets were obtained from citrated samples [29]. The cells were washed with saline and resuspended in saline to obtain $2–3 \times 10^6$ cells per ml (haemocytometer cell counts of 600–1000 cells).

Five to 50 µl of the ethanolic solution of CA or DMA were added to the cell suspensions to adjust the concentration of the reagents to $10^{-5}$ to $10^{-6}$ M. The pH was kept below 7 to prevent the hydrolysis of the anhydrides and the reaction was carried out at pH 6·4–6·9 at room remperature (ca. 18°C). Electrophoretic mobility determinations on the cells were commenced immediately after the addition of the reagents as described for polypeptide-treated cells [29]. Examination of 30–40 cells was completed within about 10 min. Mobilities were determined at intervals up to 30 min and were found to be stable. The treated cells were viable and the cell membrane was observed to be intact as judged by phase contrast microscopy (cf. 24, 25).

Suitable control samples contained ethanol in appropriate amounts to the final concentration of $10^{-5}$ to $10^{-6}$ M and were not observed to affect the cells and the cellular mobility.

*Reversible Blocking of the Cell Surface Amino Groups by DMA*

Fields [31] has studied the variation of the half-life of the breakdown of 2,3-dimethylmaleyl-glycine to 2,3-dimethylmaleic anhydride and glycine. From his plot of the half-life vs. pH (5–8), it is clear that half of the 2,3-dimethylmaleyl-glycine breaks down in about 6 min at pH 6, in about 90 sec at pH 5·5 and completely at pH 5. Based on this data very kindly made available to me by Mr. Robert Fields in advance of publication, the usefulness of 2,3-dimethylmaleic anhydride as a reversible blocking agent for the amino groups on the surface of Ehrlich ascites tumour cells, human blood platelets and lymphocytes was tested. The cells treated with DMA at pH 6·8 were examined electrophoretically and the increase in the mobility was noted, and then aliquots were washed with saline adjusted to pH 5 and 6 for the determination of mobilities at three different pH values of 5, 6 and 6·8. It must be emphasized that under these experimental conditions of pH, below 7, alkylation of the sulphydryl groups would be irreversible. The reactions in the pH range 6·4 to 6·9 have been found to be completely reversible, confirming that the alkylation of the sulphydryl groups probably does not take

place. Hence the conclusion about the reversible blocking of the cell surface amino groups is not affected and the calculations are not unduly complicated by the presence of the cell surface sulphydryl groups [15, 28, 32, 33].

## N-ETHYLMALEIMIDE (NEM)

Mobility determinations on Ehrlich ascites tumour cells (EAT) treated with NEM ($10^{-4}$ M) at pH $7{\cdot}2\pm0{\cdot}2$ were made within 3 min of the addition of the reagent at intervals up to about 20 min and were found to remain unaltered. During this short experimental time there was no evidence for the degradation of NEM at neutral pH as the optical density at 300 nm was constant [cf. 34]. The UV absorption peak at 300 nm [35] is attributed to the five-membered ring structure of NEM and any significant absorption at this peak is not exhibited by N-ethylmaleamic acid [36] which does not contain the five-membered ring structure. When NEM is hydrolysed the rupture of the five-membered ring takes place leading to the formation of N-ethylmaleamic acid. This conclusion about the lack of degradation was also supported by the thin layer chromatography experiments (solvent: chloroform: benzene: 1 : 1) performed on hydrolysed NEM (sodium salt of N-ethylmaleamic acid) and an aqueous solution of NEM (pH: 7) which had been left on the bench for about 20 min. The NEM spot ran but the hydrolysed NEM spot did not move as expected (see Fig. 2).

## RESULTS AND DISCUSSION

The data on three types of cells (EAT, human blood platelets and lymphocytes) treated with citraconic anhydride (CA) and 2,3-dimethylmaleic anhydride (DMA) and NEM are given in Table 1.

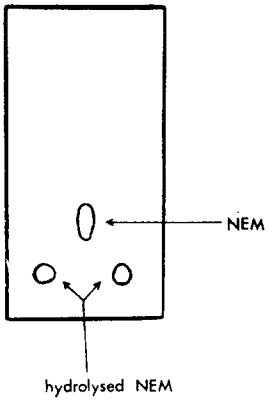


*Fig. 2. Thin layer chromatography of N-ethylmaleimide (NEM) ($10^{-4}$ M) dissolved in saline at pH $7{\cdot}2\pm0{\cdot}2$. NEM was also hydrolysed by raising the pH to 11 and heating the solution to 80°C and the pH was readjusted to $7{\cdot}2$. Solvent: Chloroform : Benzene : 1 : 1.*

The electrophoretic mobilities were converted into zeta potentials (mV) by the Helmholtz-Smoluchowski equation and the surface charge densities were evaluated by a modified form of the Gouy-Chapman equation [37] using the computer facilities as discussed in reference 29. The cells have generally been assumed to have a spheric form. From the measured diameters of the EAT cells as ($16{\cdot}9\pm9{\cdot}8$ μm) [29], that of human blood platelets as 3 μm [38] and of normal human small lymphocytes, examined under oil immersion on a wet-fixed (susa) preparation as about 6 μm [39], the surface areas of the cells were assessed. Any inaccuracy in the assessment of the surface area is not serious from the point of view of the discussion which follows. It must be remembered that the surface charge density (esu/$10^8$ μm$^2$ evaluated can be higher by a factor of up to 2, depending upon the value of the Haydon's correction factor α incorporated into the equation to account for the penetrable nature of the cell surface [37]. The zeta potentials (mV) of most cells in physiological saline are usually less than 25 mV corresponding to a mobility value of 2 μm/sec/V/cm. Provided that the zeta potentials are not too high and greater than 25 mV the equation for the evaluation of the charge density from electrophoretic measurements is reasonable and useful information about the cell surface groups may be obtained. This procedure is acceptable to most workers in the field recognizing the limitations of the procedure [37]. At present a very rigorous treatment of the equations capable of dealing perhaps fully with complex biological systems is not available.

Using the relation $4{\cdot}802\times10^{-10}$ esu=1 electron charge, from the surface charge density of the cell (esu/$10^8$ μm$^2$), the numbers of electron charges per cell surface area (μm$^2$) for the treated and untreated cells were calculated and are given in Table 1. It is clear from the Table 1 that the CA-, DMA- and NEM-treated cells exhibit an anodic mobility 20 to 30% higher compared to the suitable control samples, as would be expected by the introduction of a structure bearing a negative charge into the peripheral regions of the cells. Recalling that the electrophoretic technique determines the net charge and the adduct replaces a positive charge of the amino group on the cell surface by a negative charge (Fig. 1), the number of amino groups blocked would be half the number of the excess electron charges borne by the modified cell. The number of amino groups per cell and the actual number

*Table* 1. *Electrophoretic mobility* ($\mu m/sec/V/cm \pm s.d.$*) *of Ehrlich ascites tumour* (*EAT*) *cells, human blood platelets and lymphocytes treated with citraconic anhydride* (*CA*) ($10^{-6}$ M), *2,3-dimethylmaleic anhydride* (*DMA*) ($2 \times 10^{-6}$ M) *and EAT cells treated with N-ethylmaleimide* (*NEM*) ($10^{-4}$ M) *at* 25°. *Cell suspensions in* 0·1 45 M *NaCl buffered with* $NaHCO_3$ (0·5 N), *pH maintained at* 6·4–6·9 *for CA and DMA treatment and* $7 \cdot 2 \pm 0 \cdot 2$ *for the NEM treatment. Control samples marked A: saline; Control samples marked B: ethanol* $10^{-6}$ M *added. Number of cells examined in brackets.*

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|
| Cell type | Treated samples chemical pH | Control samples | Electrophoretic mobility ($\mu m/sec/V/cm$) | Zeta potential (mV) | Density of the *net* surface charge ($esu/10^8 \mu m^2$) | *Increase* in the net surface charge density ($esu/10^8 \mu m^2$) | *Net* electron charges per cell surface area | *Increase* in the No. of electron charges per cell | Positively charged amino groups per cell | *Actual* No. of electron charges/cell |
| Ehrlich | 6·7 | A | $-1 \cdot 08 \pm 0 \cdot 08$ (600) | 13·88 | $3672 \pm 185$ | | $6 \cdot 88 \times 10^7$ | | | |
| ascites | 6·7 | B | $-1 \cdot 05 \pm 0 \cdot 05$ (900) | 13·49 | $3567 \pm 112$ | | | | | |
| tumour§ | CA 6·7 | | $-1 \cdot 46 \pm 0 \cdot 05$ (1100) | 18·76 | $5011 \pm 79$ | 1339 | $9 \cdot 40 \times 10^7$ | $2 \cdot 52 \times 10^7$ | $1 \cdot 26 \times 10^7$ | $8 \cdot 14 \times 10^7$ |
| (mean | DMA 6·8 | | $-1 \cdot 42 \pm 0 \cdot 08$ (450) | 18·25 | $4868 \pm 189$ | 1196 | $9 \cdot 13 \times 10^7$ | $2 \cdot 24 \times 10^7$ | $1 \cdot 12 \times 10^7$ | $8 \cdot 0 \times 10^7$ |
| surface | NEM 7·2 | | $-1 \cdot 42 \pm 0 \cdot 06$ (1800) | 18·25 | $4868 \pm 80$ | 1196 | $9 \cdot 13 \times 10^7$ | $2 \cdot 24 \times 10^7$ | $1 \cdot 12 \times 10^7$ | $8 \cdot 0 \times 10^7$ |
| area: | DMA 6·8/6·0 | | $-1 \cdot 08 \pm 0 \cdot 08$ (350) | 13·88 | $3672 \pm 111$ | — | | | | |
| $900 \pm 300$ $\mu m^2$; | DMA 6·8/5·0 | | $-1 \cdot 05 \pm 0 \cdot 05$ (370) | 13·49 | $3567 \pm 79$ | — | | | | |
| ref. 29) | DMA 6·8/5·0/6·8 | | $-1 \cdot 05 \pm 0 \cdot 06$ (320) | 13·49 | $3525 \pm 182$ | — | | | | |
| Human | 6·7 | A | $-0 \cdot 91 \pm 0 \cdot 06$ (120) | 11·69 | $3078 \pm 201$ | | $1 \cdot 82 \times 10^6$ | | | |
| blood | 6·7 | B | $-0 \cdot 89 \pm 0 \cdot 05$ (300) | 11·44 | $3016 \pm 168$ | | $1 \cdot 78 \times 10^6$ | | | |
| platelets† | CA 6·7 | | $-1 \cdot 16 \pm 0 \cdot 05$ (270) | 14·91 | $3951 \pm 79$ | 873 | $2 \cdot 33 \times 10^6$ | $0 \cdot 51 \times 10^6$ | $2 \cdot 57 \times 10^5$ | $20 \cdot 77 \times 10^5$ |
| (diam. 3 $\mu m$ | DMA 6·8 | | $-1 \cdot 14 \pm 0 \cdot 05$ (120) | 14·65 | $3881 \pm 135$ | 765 | $2 \cdot 29 \times 10^6$ | $0 \cdot 45 \times 10^6$ | $2 \cdot 26 \times 10^5$ | $20 \cdot 06 \times 10^5$ |
| ref. 38: | DMA 6·8/5 | | $-0 \cdot 84 \pm 0 \cdot 05$ (150) | 10·79 | $2836 \pm 121$ | — | | | | |
| surface | DMA 6·8/5/6·8 | | $-0 \cdot 89 \pm 0 \cdot 06$ (80) | 11·44 | $3016 \pm 143$ | — | | | | |
| area: $28 \cdot 27$ $\mu m^2$) | | | | | | | | | | |
| Lymphocytes‡ | 6·8 | A | $-1 \cdot 05 \pm 0 \cdot 06$ (250) | 13·49 | $3567 \pm 112$ | | $8 \cdot 40 \times 10^6$ | | | |
| (diam. (ref. 39) | 6·8 | B | $-1 \cdot 05 \pm 0 \cdot 07$ (150) | 13·49 | $3567 \pm 119$ | | $8 \cdot 40 \times 10^6$ | | | |
| $6 \pm 0 \cdot 8$ $\mu m$;) | CA 6·8 | | $-1 \cdot 28 \pm 0 \cdot 07$ (280) | 16·45 | $4372 \pm 120$ | 805 | $10 \cdot 29 \times 10^6$ | $18 \cdot 95 \times 10^5$ | $9 \cdot 47 \times 10^5$ | $9 \cdot 34 \times 10^6$ |
| surface area: | DMA 6·8 | | $-1 \cdot 28 \pm 0 \cdot 09$ (120) | 16·45 | $4372 \pm 141$ | 805 | $10 \cdot 29 \times 10^6$ | $18 \cdot 95 \times 10^5$ | $9 \cdot 47 \times 10^5$ | $9 \cdot 34 \times 10^6$ |
| $113 \pm \mu m^2$ | DMA 6·8/5/6· | | $-1 \cdot 08 \pm 0 \cdot 08$ (80) | 13·88 | $3672 \pm 235$ | — | | | | |
| (ref. 29). | | | | | | | | | | |

*For computer processing of the electrophoretic measurements for calculating the electrokinetic parameters, standard deviations, standard error of the mean of the mean values and the $p$ values from Student's $t$-test see reference [29]. Standard deviations for the values of electrophoretic mobility and the surface charge density are printed; $p$ values $< 0 \cdot 001$.

†4 individuals (G,M,W,Z). ‡3 individuals (G,M,W). §24 Albino Tuck-TT tested, 2–3 month old male mice bearing Ehrlich ascites tumour: cells harvested 6–8 days after transplantation.

N.B.—pH: 6·8/5/6·8 etc. indicates that the cell suspension had been subjected to these changes in pH successively before the electrophoretic examination was carried out.

of electron charges per cell, being the sum in columns 8 and 10 were calculated (see columns 10 and 11 of Table 1).

The test for the reversibility of the blocking of the cell surface amino groups showed that the electrophoretic mobility of the cells on treatment with DMA increased at pH 6·8. After these chemically-modified cells had been washed with saline adjusted to pH 5 and 6, the mobility at pH 5, 6 and 6·8 was found to return to the control value as expected from the data on the breakdown of 2,3-dimethyl-maleyl-glycine (Table 1).

In view of the present findings that on the surface of EAT cells positive charges of the amino groups number $11{\cdot}9\times10^6$ per cell surface area (with a median value of 900 $\mu m^2$ over the range 600 to 900 $\mu m^2$) it is interesting to examine the findings of Straumfjord and Hummel [40] on Sarcoma 180 cells. In a representative experiment these authors found that for the cells (measured mean diam. 18·3 $\mu m^2$; mean surface area of 1052 $\mu m^2$) treated with 0·006% polyxenyl phosphate-labelled P (PXP-32) for 12 hr at pH 7·3, phosphate buffer of ionic strength 0·173, the uptake of P-32 corresponded to $3{\cdot}97\times10^{-10}$ mg per cell. Of the total PXP-32 taken up only 18% was held on the surface as determined by cell electrophoresis. These authors do not give enough details to be able to allow a calculation of the molarity of the solutions. However, for a polymer of a molecular weight of about 6000 [41], it is perhaps more convenient to calculate and visualise the number of molecules of the polyanions bound to the cell surface as $7{\cdot}3\times10^6$ molecules of PXP per cell surface area of 1052 $\mu m^2$. For a purely qualitative analysis it is not too unreasonable to assume that the electrokinetic makeup of the Sarcoma cells is similar to that of the EAT cells and that the Sarcoma 180 cell surface also bears approximately $12\times10^6$ positively charged amino groups, as the EAT cells, onto which 5 to $14\times10^6$ molecules of the polyanion (PXP) bind [40] as expected. Unfortunately, more precise data on the effect of PXP-treated EAT cellular mobility are not available.

## N-ETHYLMALEIMIDE (NEM)

The results on NEM-treated EAT cells obtained about three years ago had also shown an increase of about 22% in the electrophoretic mobility but had not been published because NEM is not specific for thiols as it had been reported to react with the imino groups of the imidazole derivatives and amino groups of peptides [26, 27]. It should be pointed out that under the experimental conditions outlined in the NEM experiments being reported in the present paper, NEM is unlikely to react with simple alpha amino groups of a chemical as studied by Smyth *et al.* [26, 27]. However, the agreement between the values of the electrophoretic mobility of the EAT cells treated with CA, DMA and NEM and the calculated number of the positive charges on the EAT cell surface in the three cases (Table 1, column 8) is so striking that the possibility must be borne in mind that protein/peptide amino groups *are* able to react in some biological systems (Fig. 3).

## HUMAN BLOOD PLATELETS

The present electrokinetic data on CA- and DMA-treated platelets given in Table 1 show that the number of the positive charges due to the amino groups on an intact platelet is $2{\cdot}57\times10^5$ to $2{\cdot}26\times10^5$. From electrokinetic data on aldehyde-treated platelets [17], the number of charges has been calculated to be $3{\cdot}5\times10^5$ [42]. If groups other than amino are blocked by aldehyde treatment or additional positively charged groups are exposed following prolonged treatment with severe reagents, the number of groups calculated from the electrokinetic data on cells modified in this manner could well yield the numbers of groups on cells much higher than calculated in the present experiments.

### *Surface Charges and Aggregation of Platelets*

It is interesting to consider the significance and any possible involvement of the cell surface amino group-generated positive charges ($2{\cdot}26$ to $2{\cdot}57\times10^5$ per platelet) in connection with platelet aggregation caused by anionic polymers and other charged clumping agents [29, 43], particularly by ADP [44–46], and ADP-induced changes in the electrophoretic mobility of platelets [47, 48]. Born's calculations [49] showed that the number of ADP-reacting sites on a human platelet is $2\times10^5$ and the other electrokinetic data [47, 48] showed that $1{\cdot}17\times10^5$ molecules of ADP per platelet caused a small but significant change in the platelet mobility. The correspondence between the number of reacting sites from the other references quoted [47–49] is indeed striking. Born's hypothesis for the action of ADP proposes that ADP forms very short-lived complexes with a component of the platelet membrane, as it has been assumed, for example, for the action of acetylcholine on a smooth muscle membrane [50]. The evidence against the direct and lasting adsorption

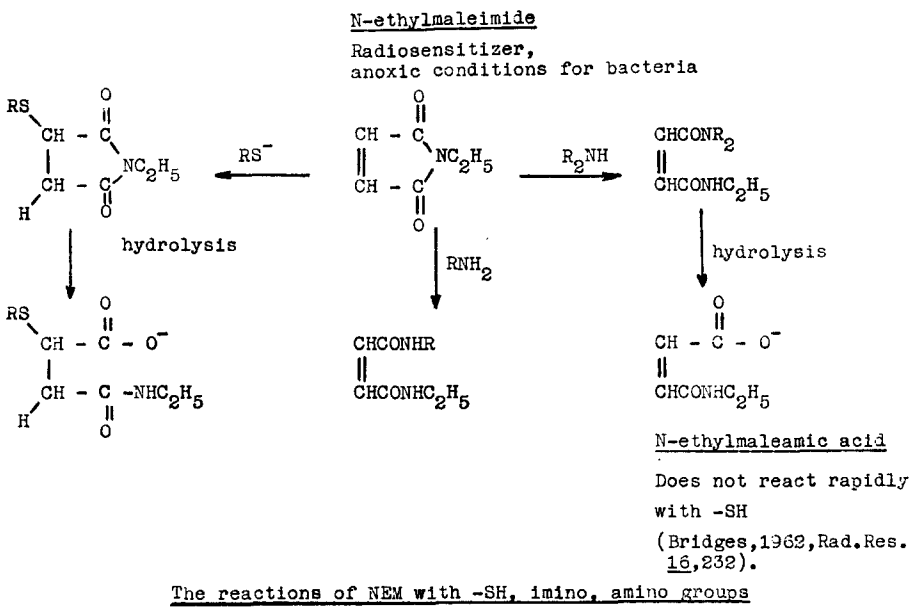


*Fig.* 3. *The reactions of imino, amino and sulphydryl groups with N-ethylmaleimide* [26, 27, 54, 56].

of ADP on the platelet surface as discussed in reference [42] perhaps seems formidable but in relation to this proposal, arguments that platelets do not bind ADP [51] are invalid because they depend on the meaning of binding in terms of time as already pointed out by Born [52]. The possibility of a primary interaction between the platelet surface amino group-generated positive charges and the negatively charged substances such as ADP, *initiating* configurational changes in the cell surface ultrastructure *before* secondary changes become apparent, as proposed by Born [49, 52], seems real.

The surface of thrombocytes is mainly protein in nature as indicated by the pH-mobility relationships [16, 17]. The structural proteins of the cell surface have unsatisfied electrostatic forces around the various charged groups and when these forces are disturbed by mopping up the cell surface amino group-generated positive charges following interaction with the negatively charged ADP, the instability of the ultrastructure is initiated and the platelets aggregate. Szent-Györgi has given an excellent discussion of this idea in his latest book [53].

Similarly, the presence of the cell surface amino groups must be considered while discussing the inhibition of ADP-induced changes of platelet mobility by NEM and the reversal of it by cysteine [47]. The importance of the various cell surface groups in these phenomena have been discussed in greater detail elsewhere [43, 54, 55].

## LYMPHOCYTES

The electrophoretic mobility of human lymphocytes (small; mean diam $5{\cdot}8 \pm 0{\cdot}8$ μm) treated with CA and DMA at pH 6·8 was found to be $-1{\cdot}28 \pm 0{\cdot}05$ μm/sec/V/cm and is nearly the same as that of untreated lymphocytes in the high region of pH (ca. 10·5) as would be expected when the positively charged groups of high pK of the cell surface proteins are charged fully [18, 19]. The number of amino groups blocked by CA and DMA was found to be the same: $9{\cdot}47 \times 10^5$. Since the net charge density gives a value of $8{\cdot}4 \times 10^6$ electron charges per cell, the total number of electron charges on the surface of normal human small lymphocyte works out to be $9{\cdot}34 \times 10^6$ with an upper limit of $18{\cdot}68 \times 10^6$ (Table 1).

## CONCLUSION

Although the electrokinetic behaviour of EAT cells, lymphocytes and platelets at high pH values [12, 15–18, 38] has been found to be reversible during the short experimental time before any deleterious effects may have occurred, some valid criticisms about the data obtained at extremes of pH values can be made. The conformational changes of the ultrastructure, further ionization of the cell surface groups and denaturation of the cell surface must be considered. It is also clear that there is a need for caution in the use of NEM for the detection of —SH groups/compounds in mixtures derived from biological sources [24–27, cf. 47, 54, 56]. The present

studies demonstrate that under suitably mild conditions, CA and DMA at pH 6·4 to 6·9 at room temperature can be used to examine the presence of amino groups on the surface of cells without subjecting them to drastic experimental conditions which could cast a doubt on the usefulness of the electrokinetic data to obtain information about the molecular architecture of normal and cancer cells.

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**Note added in proof**—To ascertain whether DMA interacted with the cell surface —SH groups [33] under the experimental conditions of pH and temperature employed in the experiments on live, intact cells, the following experiments were carried out. Cysteine and glutathione, dissolved in saline, were allowed to react with DMA at various values of pH [5–9] at room temperature and subsequently with the specific —SH blocking agent 6,6′-dithiodinicotonic acid (CPDS) [33] to estimate the —SH content. No changes in —SH content were observed up to pH 8·5. The details will be published elsewhere [55]. It was concluded that DMA does not react with —SH groups up to pH 8·5, or that the reaction is extremely slow. At pH 9 the interpretation of the u.v. absorption spectra becomes difficult because of the pH effects on the ionization of —SH. Fortunately the usefulness of DMA for the reversible blocking of amino groups on cell surfaces is not in doubt and the method can be recommended with confidence.

## SUMMARY

*On the surface of Ehrlich ascites tumour (EAT) cells, human blood platelets, and lymphocytes, various ionizable groups of high pK (amino and sulphydryl) are present but their proper characterization had proved extremely difficult without resorting to severe chemical treatment. The present work describes the development of a suitably mild chemical treatment of intact live cells using citraconic anhydride (CA) and 2,3-dimethylmaleic anhydride (DMA) at pH 6·4 to 6·9 at room temperature (ca. 18°C) to block the cell surface amino groups reversibly. From electrokinetic data on chemically-modified cells, it was possible to calculate the numbers of positively charged amino groups on the cell surface without undue complications from the presence of the cell surface sulphydryl groups. The reaction of the sulphydryl groups with the reagents under the experimental conditions is irreversible. When the positively charged amino groups are blocked, the anodic mobility of the treated cells increases by about 20% because of the elimination of the positive charges and also because the adduct introduced into the cell periphery, carrying an ionizable carboxyl group, becomes operative in the electrophoretic plane of shear. If the treated cells are washed with saline of pH 5 or 6, the adduct is split off and the mobility of the cells returns to the control value thus showing that the blocking of the amino groups is reversible and that the calculations are not complicated by any involvement of the cell surface sulphydryl groups. Treatment of the tumour cells with N-ethylmaleimide, which is not specific for thiols, increases the mobility nearly to the same extent as with the two anhydrides, CA and DMA, suggesting that perhaps all the three reagents (CA, DMA and NEM) block the same sites. From the electrokinetic data the number of positively charged amino groups and the actual number of electron charges on the surface of the three cell types (Ehrlich ascites tumour cells, human blood platelets and lymphocytes) have been calculated and discussed in relation to their significance for the adhesion and aggregation phenomena.*

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# Plasma Thyrotropin (TSH) and Long-Acting Thyroid Stimulator (LATS) in Patients Treated for Thyroid Carcinoma

L. VALENTA, T. LEMARCHAND-BÉRAUD and A. VANNOTTI
*Département de Biochimie Clinique, Clinique Médicale Universitaire, Lausanne, Switzerland*

## INTRODUCTION

IODINE concentration and organic binding is only rarely found in thyroid carcinoma where there is a normal thyroid gland (Fig. 1). Total thyroidectomy (Fig. 2) or radiothyroidectomy (Fig. 3) is usually necessary to "induce" iodine accumulation in metastatic thyroid carcinoma, and is used as an introduction to radioiodine therapy [1–3].

However, any treatment decreasing the thyroid hormone supply may stimulate the growth of metastatic thyroid carcinoma [4]. Stimulation of function and growth are probably due to the increase of the thyrotropic hormone (TSH) in the blood. Due to the negative thyroid-pituitary feed-back mechanism the increased level of thyrotropin results from the decreased amount of circulating thyroid hormone.

To study the role of thyroid-stimulating substances in thyroid carcinoma, the level of thyrotropin (TSH) has been repeatedly determined in the plasma of ninety patients during the treatment, and compared with the values obtained in eighty-three normal euthyroid subjects. In 22 of these cases the long-acting thyroid stimulator (LATS), which is found in the blood of thyrotoxic patients, was also examined [5].

## MATERIAL AND METHODS

Diagnosis of thyroid carcinoma was confirmed histologically in all cases. Thyroid function was determined by correlation of clinical and laboratory findings, including tests for protein-bound iodine, butanol- extractible iodine, free thyroxine [6], and radioiodine accumulation and excretion rate, scintigraphy, stimulation and suppression tests [7].

The treatment of metastatic thyroid carcinoma consisted in total or subtotal thyroidectomy, and ablation of a maximum possible cancerous tissue. When surgical thyroidectomy was only subtotal and carcinoma function was absent, radiothyroidectomy was performed. After the occurrence of radioiodine accumulation in the cancer, repeated therapeutic doses of $^{131}I$ were administered according to the method of Šilink *et al.* [8] until destruction of functioning tissue. Hypothyroidism resulting from this therapy was then corrected by exogenous thyroid hormone administration.

The plasma of the patients was stored at −20°C till the time of the assay. Thyrotropin was determined by radioimmunoassay using bovine TSH standard as a reference [9]. The results expressed in USP units were analyzed by the Student *t*-test. Values obtained with bovine TSH standard are approximately tenfold compared to the results of radioimmunoassay using human TSH standard.*

The long-acting thyroid stimulator was detected by bioassay in mice [10]. LATS was

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*Arbitrary activity of human TSH, standard A, from National Institute of Medical Research, Mill Hill, London, is 50 mU/33 μg.

considered positive, when, 9 hr after the injection, the tested serum increased the blood radioactivity of experimental animals to more than 150% of the initial value, and when the 9 hr value exceeded the 2 hr value.

## RESULTS

The results of TSH tests are summarized in the table. The value found in 83 normal euthyroid subjects was 0·19±0·03 mU/ml.

Compared to the normal level, TSH was significantly higher in the plasma of thyroid-carcinoma patients before any treatment ($P$=0·001). Further increase of TSH was observed after total thyroidectomy, but not after subtotal thyroidectomy. Maximum increase was found from the fourth to the sixth week after the operation and was followed by a subsequent decrease. This decrease of the TSH level was encountered even when there was no functioning thyroidal or carcinomatous tissue. An illustrative case is given in Fig. 4.

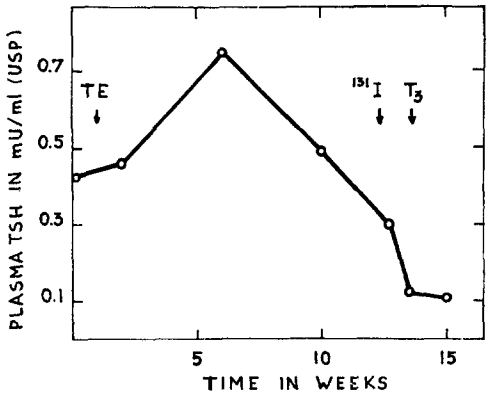


*Fig. 4. Plasma-TSH changes after total thyroidectomy in a patient suffering from metastatic thyroid carcinoma. Compared with normal subjects, TSH is high before any treatment and increases further after total thyroidectomy. It later returns to values similar to those observed before treatment. TSH decrease to normal value follows triiodothyronine (T3) therapy which even prevented possible TSH increase after 131I therapy.*

After radioiodine treatment variations in plasma TSH level were observed in many cases with a drop to normal values in the second or third week after $^{131}$I administration. This minimum coincided with the clinical symptoms of hyperthyroidism which often occurs after radioiodine therapy, and which are probably due to the release of thyroid hormones from the tissue destroyed by radiation. Occasionally, an initial decrease of the plasma TSH was observed on the first day after radioiodine administration. An illustrative case is shown in Fig. 5.

In 10 out of 14 cases maximum increase of TSH after radioiodine therapy was observed

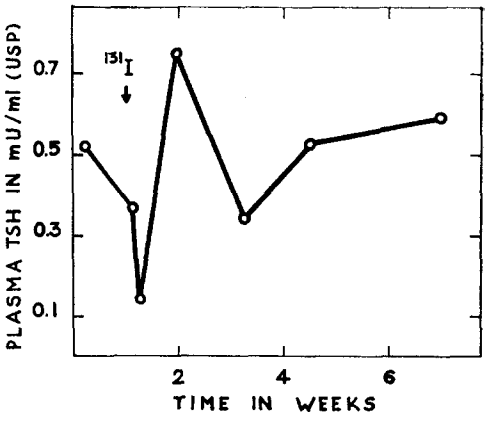


*Fig. 5. TSH variations after 137 mc of 131I administered to a patient suffering from functioning thyroid carcinoma. A drop in plasma TSH is observed during the first day and another during the third week after radioiodine therapy. Total thyroidectomy preceded radioiodine treatment, however, the functioning thyroid carcinoma provoked hyperthyroidism. In spite of this the initial TSH value is high.*

from the fourth to the sixth week after $^{131}$I administration. This TSH increase was greater than that after total thyroidectomy, and in two cases exceeded 10 mU/ml. When radioiodine treatment was applied shortly after total thyroidectomy, the TSH increase was less pronounced or even absent. The high values did not persist for a long time and decreased to low elevation levels even when there was no functioning tissue.

Where there was presence of functioning carcinoma, the maximum TSH increase after thyroidectomy (radiothyroidectomy) was never as high as with non-functioning carcinoma. In euthyroid patients possessing functioning thyroid carcinoma only, without the normal thyroid gland, plasma TSH was slightly elevated.

Exogenous thyroid hormone (triiodothyronine or thyroxine) was administered in order to correct or prevent hypothyroidism resulting from combined radioiodine treatment and surgery. Administration resulted in a TSH decrease starting on the first day of therapy and reaching normal values at the end of the first week, before hypothyroidism had been corrected. However, in the group of patients treated with exogenous thyroid hormone for several months, the average TSH value was significantly higher than normal ($P$=0·001).

Interruption of hormonal therapy in patients without functioning thyroidal tissue led to a rapid increase of TSH before development of clinical and laboratory hypothyroidism. Maximum values occasionally exceeding 10 mU/ml were observed at the end of the first week after stopping treatment. These high values were transitory and a value 0·50 mU/ml

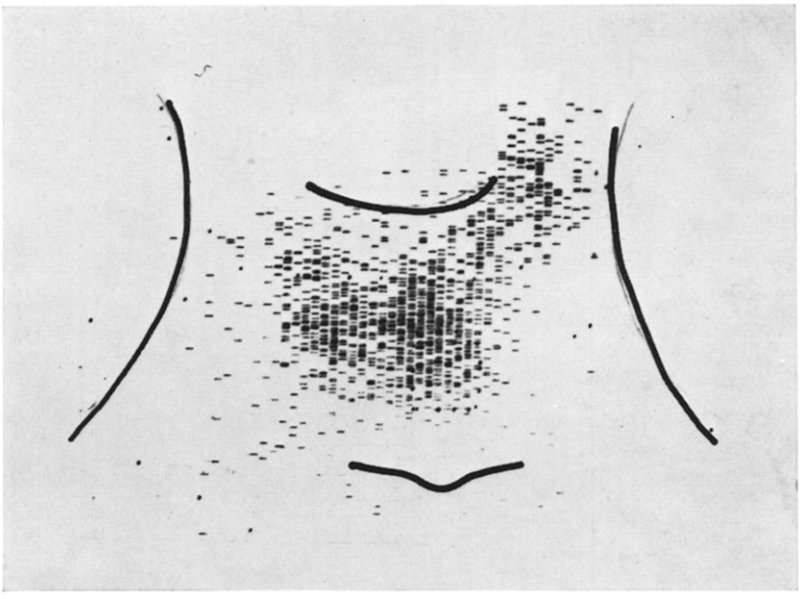

*Fig.* 1. *Scintigram* 24 *hr after administration of a diagnostic dose of radioiodine. Radioiodine is concentrated in the thyroid gland and in a lymph node metastasis of thyroid carcinoma under the angle of the mandible to the left.*

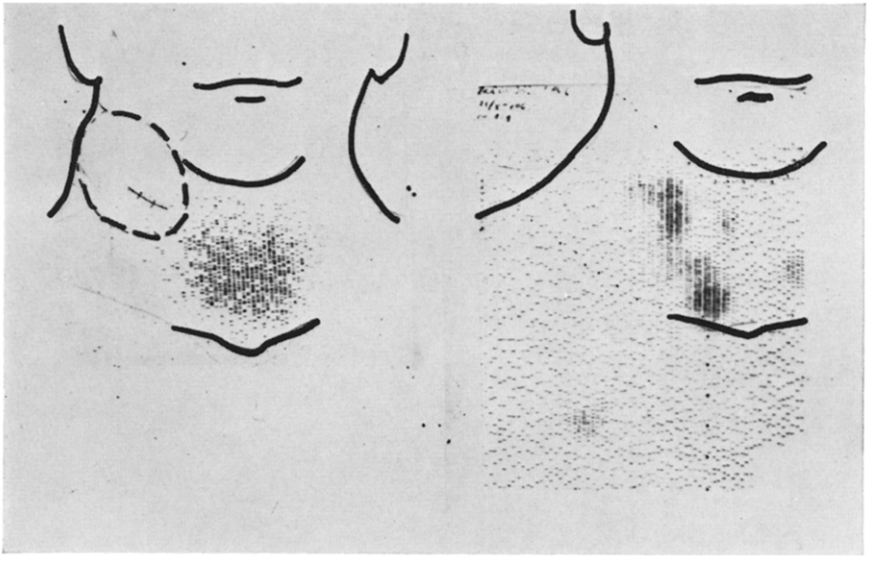

*Fig.* 2. *Scintigram obtained* 24 *hr after* $^{131}I$ *administration:*

(*a*) *before any treatment the radioiodine is only concentrated in the thyroid gland.*

(*b*) *after total thyroidectomy there is radioiodine accumulation in the thyroid region, in the course of the ductus thyroglossus, in the recurrent lymph node metastasis of thyroid carcinoma on the right, and in a small metastasis on the right of the chest, which was not located by radiography.*

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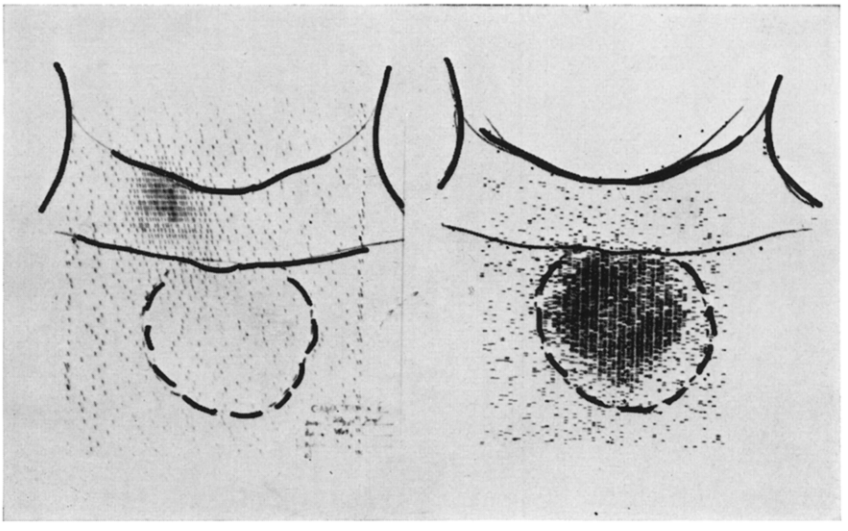

*Fig.* 3. *Scintigram obtained in a patient:*

(*a*) *after subtotal thyroidectomy.*

(*b*) *after subsequent radiothyroidectomy. The bone metastasis of thyroid carcinoma in the manubrium sterni begins to accumulate radioiodine only after total destruction of the thyroid by* $^{131}I$ *therapy.*

*Table* 1. *Plasma-TSH levels in patients with thyroid carcinoma and the effect of treatment*

| Treatment | No. of cases | Mean TSH value (mU/ml plasma) B-TSH USP reference standard ± : standard deviation | Probability that the mean differs from the normal subjects |
|---|---|---|---|
| Before any treatment | 33 | 0·31±0·05 | $P=0·001$ |
| Total thyroidectomy | 31 | 0·84±0·38 | $P=0·001$ |
| Subtotal thyroidectomy | 22 | 0·25±0·035=0·04 | N.S. |
| Radioiodine treatment | 21 | 1·27±0·54 | $P=0·001$<br>$P=0·05$ if compared to the group before treatment |
| Exogenous thyroid hormone (less than one month) | 19 | 0·18±0·03 | N.S.<br>$P=0·05$ if compared with the group before treatment |
| *Ibid.*, several months | 32 | 0·27±0·03 | $P=0·001$ |
| Total of patients studied | 90 | | |
| Normal euthyroid subjects | 83 | 0·19±0·03 mU/ml | |

was found in two cases where hypothyroidism was not corrected for several months.

The long-acting thyroid stimulator (LATS) was found in the plasma of 10 out of 22 examined patients. Four patients with positive LATS suffered from hyperthyroidism. The mean value of LATS in the 10 positive cases was 242±51% after 9 hr. In two cases treated with prednisone a decrease in LATS values was observed, along with an improvement of the disease.

## DISCUSSION

The reported results of TSH changes during the treatment of thyroid carcinoma indicate that the feed-back mechanism is preserved in carcinoma patients and is realized not only between the pituitary and thyroid gland, but also between the pituitary and thyroid carcinoma. Any treatment decreasing the quantity of thyroid hormone, secreted either by normal or malignant thyroid tissues, resulted in an increase of plasma TSH. This occurred after total thyroidectomy and radioiodine treatment, and also after discontinuing the exogenous thyroid hormone administration in a patient without thyroid tissue. In all these cases there was a temporary maximum increase, which was probably due to release of TSH stored in the pituitary. For a period of time after exhaustion of the stores, the pituitary was unable to react with the same intensity to a new stimulation. This could be deduced from the absence of TSH increase after radioiodine therapy where the latter was administered shortly after total thyroidectomy.

Some peculiarities of the reported TSH changes suggest that the feed-back mechanism in carcinoma patients is modified: (1) Plasma TSH was higher than normal before any treatment, which is in agreement with the findings of other authors [11, 12]. (2) It was also higher in patients whose functioning carcinoma maintained euthyroidism or produced hyperthyroidism [13], while in thyrotoxic patients plasma TSH levels are low [14]. (3) Exogenous thyroid hormone administered for several months failed to totally inhibit the TSH secretion, as is the case in non-cancerous hypothyroid patients [15]. (4) The TSH increase preceded clinical and laboratory hypothyroidism.

The cause of these peculiarities remains unexplained. Hyperplasia of pituitary thyrotrophs secreting TSH was found in some thyroid carcinoma patients [16]. This hyperplasia could be primary, or secondary, to chronic suppression of the function of the normal thyroid gland containing carcinoma. Such a suppression probably occurs, due to expansive tumor growth and to latent changes in the biochemistry of the thyroid tissue. Indeed slightly decreased thyroglobulin-iodine content, or increased proteolytic activity, was

encountered in some cases in the thyroid tissue surrounding thyroid carcinoma (unpublished results).

Proliferation of thyroid carcinoma tissue under the influence of increased TSH sometimes exceeding 10 mU/ml is most probable. Since the high TSH values follow acute withdrawal of thyroid hormone supply, and since they precede the clinical development of hypothyroidism, preventive exogenous thyroid hormone medication is to be recommended in all types of treatment limiting thyroid function, e.g. after total thyroidectomy or immediately after radioiodine administration. In long-term treatment exogenous thyroid hormone in doses correcting hypothyroidism is often insufficient to suppress plasma TSH to normal value. Therefore, maximum tolerated doses are recommended, which could be efficient.

Positive LATS activity was frequently observed in the group of our patients. This could result from the selection of our cases which include carcinoma causing hyperthyroidism. Decrease of LATS positivity, along with improvement in the disease, was observed in some of our cases following prednisone administration. Thus the LATS finding may be of practical value in indicating Prednisone therapy.

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## SUMMARY

*Thyroid stimulating hormone (TSH) determined by radioimmunoassay, and long-acting thyroid stimulator (LATS) determined by bioassay were followed in the plasma of ninety patients treated for thyroid carcinoma. Before any treatment plasma TSH was significantly higher (0·31 mU/ml) than in 83 normal euthyroid subjects (0·19 mU/ml). It increased further after total thyroidectomy and radioiodine treatment but not after subtotal thyroidectomy. When hypothyroidism resulting from therapy of the metastatic thyroid cancer was corrected with exogenous thyroid hormone, decrease of plasma TSH was observed during the first week of treatment. However, after administration for several months, TSH increased to similar levels as before treatment, even when hypothyroidism has been corrected with suitable thyroid hormone treatment. LATS was positive in 10 of 22 cases. Its incidence was especially high in patients suffering from hyperthyroidism due to thyroid carcinoma.*

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# Observations on the Fine Structure of Human Ureteric Tumours

BOJAN FLAKS*, EDWARD H. COOPER and JUDY C. KNOWLES

*Department of Experimental Pathology and Cancer Research, School of Medicine, Leeds 2, Great Britain*

PRIMARY carcinomata of the ureter are comparatively rare tumours in man and domestic animals. It has been estimated that the relative incidence of carcinoma in different parts of the urinary tract is 10 : 1 : 40 for the renal pelvis, ureter and bladder respectively [1]. There are a few descriptions of the ultrastructure of the ureteric epithelium in laboratory animals [2] but there do not appear to be any corresponding studies in man. There is also a paucity of information of the ultrastructure of tumours of the renal tract. Battifora and his colleagues [3] have described some ultrastructural features of transitional cell carcinoma of the bladder in man, but even these commonplace tumours seem to have attracted little attention. Transitional cells have many special, functional properties and carcinomata arising in these epithelia have a wide spectrum of organisation, extending from very well differentiated papillary to solid anaplastic growth [4].

In this paper we describe these carcinomata of the ureter and have compared the ultrastructure of these tumours with "normal" ureteric epithelium. It can readily be appreciated that in man it is very difficult, for obvious ethical reasons, to obtain fresh normal ureters for study. If necropsy specimens are used, gross artefacts can be induced by post-mortem changes. The surgical specimens that have been used in this study were chosen from non-malignant cases in which there was only minimal ureteric epithelial hyperplasia.

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*Present address: Department of Pathology, University of Bristol.

In one of the patients the tumours contained large cytoplasmic structures that are probably formed from aggregated ribosomes, which do not appear to have been previously described.

## MATERIAL AND METHODS

*Clinical details of tumours*

*Case* 1. *J.S. Aged* 54. One-month history of haematuria and a non-functioning kidney revealed on X-ray. Two carcinomata 7 cm apart were found in the ureter at operation.

*Pathology.* The lower tumour was a well differentiated transitional cell carcinoma (TCC) showing only focal invasion of the fronds. The upper tumour was a solid TCC invading into the ureteric muscle.

*Case* 2. *G.E. Aged* 66. Recurrent bladder papillomata for several years before developing an extensive bladder tumour and a ureteric tumour which were treated by total cystectomy and ureterectomy.

*Pathology.* The ureteric tumour was a poorly differentiated TCC with invasion into the base of the tumour. The bladder tumour was a solid TCC, deeply invading into muscle. In all aspects the ureteric tumour was less well differentiated than either tumour in Case 1.

*Preparation of specimens*

Portions of normal and tumour tissues were placed in ice-cold fixative and subdivided into one mm cubes, to allow rapid penetration of the fixative. The tissue was fixed for a total of 4 hr at 0–4°C in 5% glutaraldehyde, buffered at pH 7·2 with 0·067 M cacodylate buffer [5, 6]. The blocks were then washed for a minimum of 16 hr at 0–4°C in 0·25 M sucrose in 0·1 M cacodylate buffer and post-fixed in

Millonig's phosphate-buffered 1% osmium tetroxide [7] at 0–4°C for 2 hr. Rapid dehydration in ethanol was carried out at room temperature and the blocks were embedded in Epon 812 [8]. Thin sections, cut with glass knives on a Sorvall MT-1 ultramicrotome, were picked up on naked copper grids, stained with lead tartrate [9] and examined in an RCA, EMU-3G electron microscope.

## RESULTS

Electron microscopy of both normal and tumour epithelium revealed evidence of some mechanical damage at the free surface of the tissues. Although the internal fine structure of the surface cells was well preserved, these cells were frequently ruptured; in the tumours, part of this damage was probably the result of the intrinsic ageing and sloughing of the tumour surface cells. The present observations are confined, therefore, mainly to the deeper layers of the tissues examined.

*Normal ureter*

The normal ureteric epithelium, several layers thick, rested on a base of amorphous material in which was embedded a moderate quantity of collagen. A definite basement membrane was present. The cells of the basal areas were irregularly rounded in outline and loosely packed, the area of cell contact being extended by interdigitating cytoplasmic processes (Fig. 1a). The cell surfaces of adjacent cells were closely apposed. Desmosomes were rarely observed. In the more superficial areas of the epithelium, the cells exhibited varying degrees of separation. Wide intercellular spaces were present and the cells had irregular microvillous surface processes corresponding to the interdigitating processes observed in the basal cytoplasm which projected into these spaces. There was, however, little significant variation in the internal fine structure of cells at various levels of the epithelium (Fig. 1b). The nuclei were roughly oval in profile, frequently possessing a single deep invagination and containing a single small nucleolus.

The cytoplasm contained a moderate amount of glycogen and small spherical mitochondria, as well as a limited quantity of the granular endoplasmic reticulum, in the form of small cisternae. A large Golgi zone was present in the perinuclear region of the cytoplasm. Lysosomes were occasionally present.

*Ureteric tumours*

*Case* 1. The two tumours obtained from Case 1, although differing histologically, exhibited no significant ultrastructural differences. The following observations, therefore, apply equally to both tumours.

These tumour cells were greatly elongated in the basal areas of the epithelium, in a direction perpendicular to the surface, becoming more compact in shape in the superficial regions (Fig. 2). Their relation to the amorphous base material and to one another resembled that of the normal ureteric epithelial cells. However, the tumour cells and their nuclei were enlarged relative to the normal cells. The nuclei frequently contained large, densely stained nucleoli which exhibited the type of microsegregation of their constituents which has been described in hepatic neoplasia [10] (Fig. 3a). The nucleoplasm presented a coarse appearance owing to the irregular clumping of the chromatin.

In comparison to that of the normal cells the tumour cell cytoplasm contained less glycogen and the Golgi zones were greatly reduced in size (Fig. 3b) while the mitochondrial population was similar, both numerically and morphologically, loss of cristae being observed infrequently. The granular endoplasmic reticulum was present in normal quantity although occasional bizarre forms were found. Pairs of closely apposed cisternae were observed which lacked attached ribosomes on their adjoining surfaces. These appear to be identical to similar structures described in HeLa cells [11] and in anaplastic hepatomata [12]. Increased numbers of free ribosomes, arranged in rosette-like polysomal aggregates, were observed. Moderately increased numbers of lysosomes were present in many of the tumour cells.

A constant feature of the tumour cell cytoplasm was the presence of extensive tracts of fibrillae. These were usually localized in such a manner as to surround the nucleus closely, other cytoplasmic constituents often being displaced towards the cell periphery. The tracts of fibrillae usually extended from their perinuclear sites towards the apical and basal regions of the cytoplasm. This arrangement was most apparent when the cell polarity was clearly defined, as in the basal areas of the tumour epithelium.

A small percentage of tumour cells from both the tumours obtained from Case 1 possessed a single, large, dense, ribonuclear body (Fig. 4–6). These bodies were branched and did not possess a limiting membrane. In high magnification electron micrographs they appeared to consist of closely packed particles, identical in size and density to ribosomes. Their

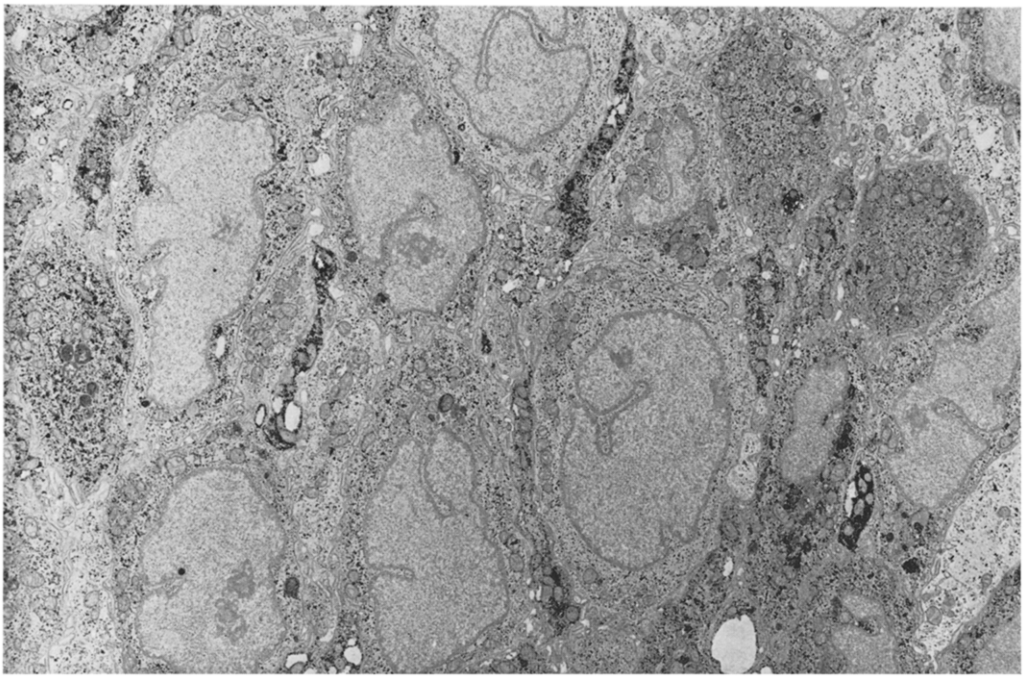

*Fig.* 1(*a*). *Ureteric epithelium showing closely packed cells; indented nuclei with mesh-like nucleolus. Heavy glycogen deposits in cytoplasm; sparse endoplasmic reticulum,* × 5136.

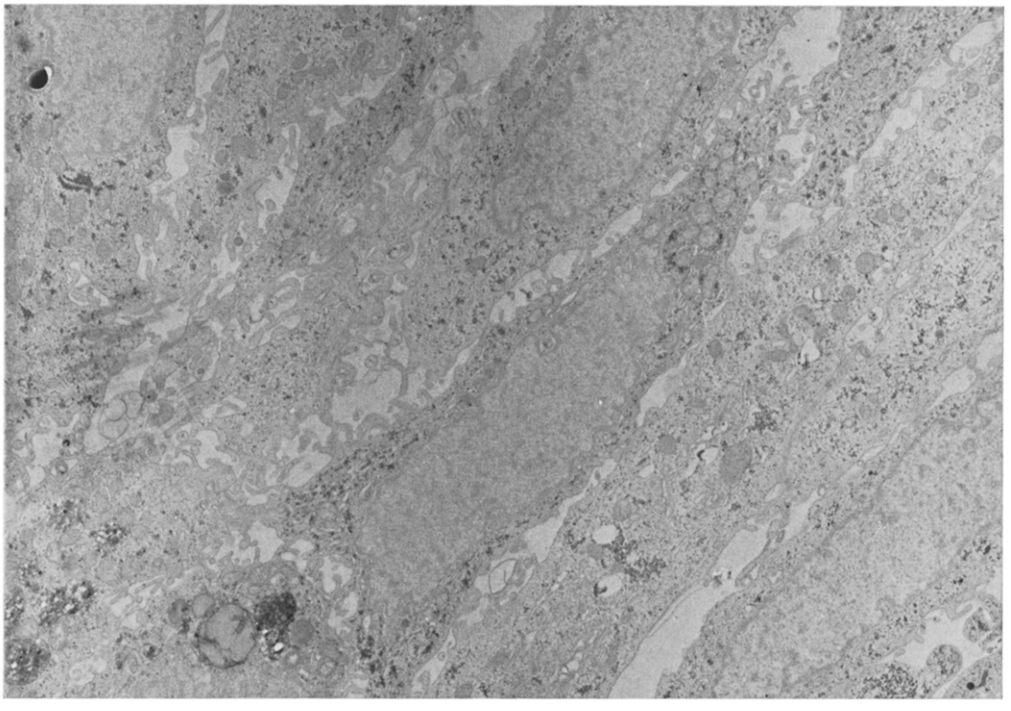

*Fig.* 1(*b*). *Ureteric epithelium from an area of mild hyperplasia, showing very loose packing of cells with marked, surface microvilli,* × 6770.

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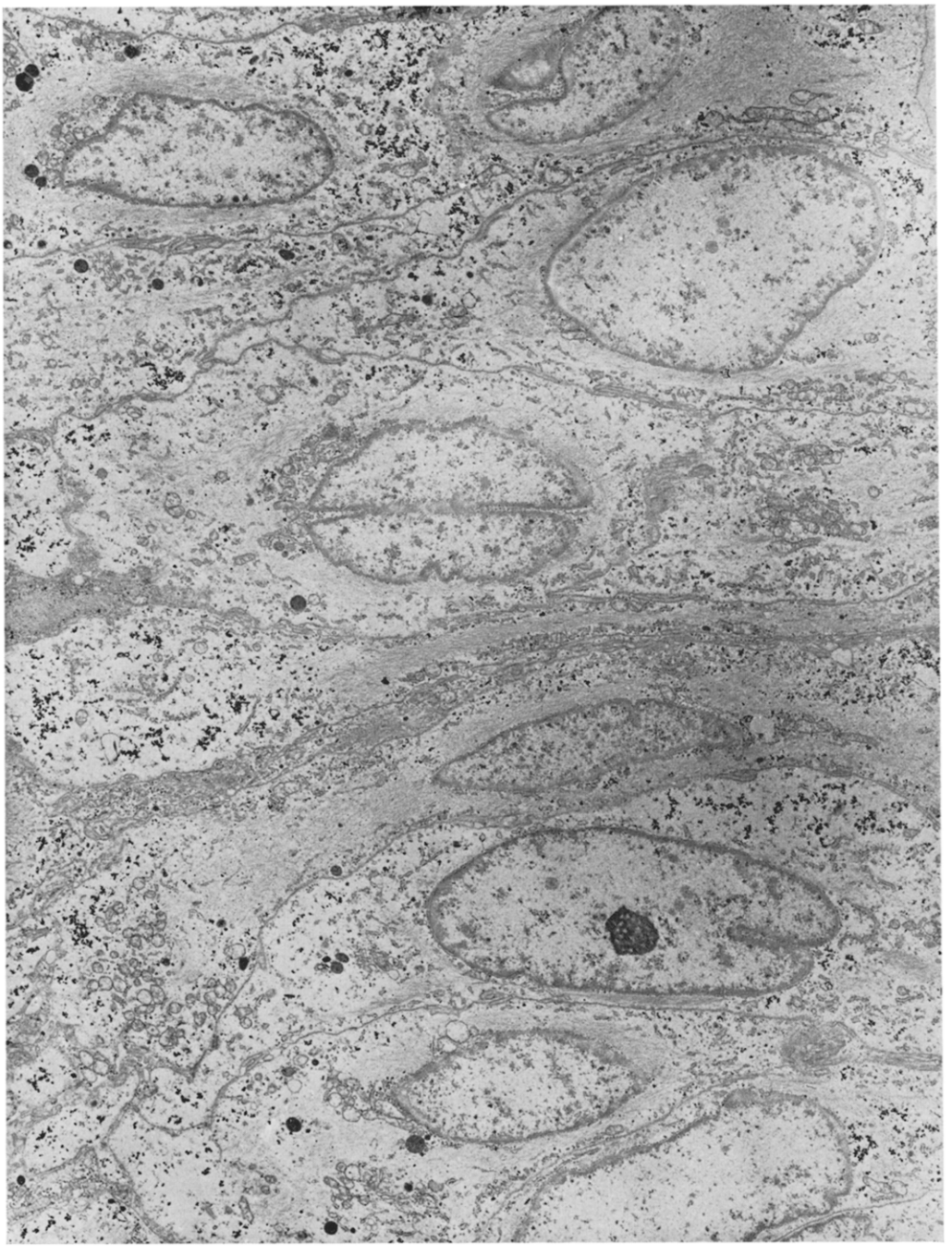


*Fig.* 2. *T.C.C. of ureter* (*Case* 1): *base of papillary frond. Regular elongated cells, dense meshwork nucleolus and perinuclear fibrillary material. The mitochondria in this area of the tumour appear to be grouped in clumps, leaving large parts of the cytoplasm relatively free of organelles,* × 3542.

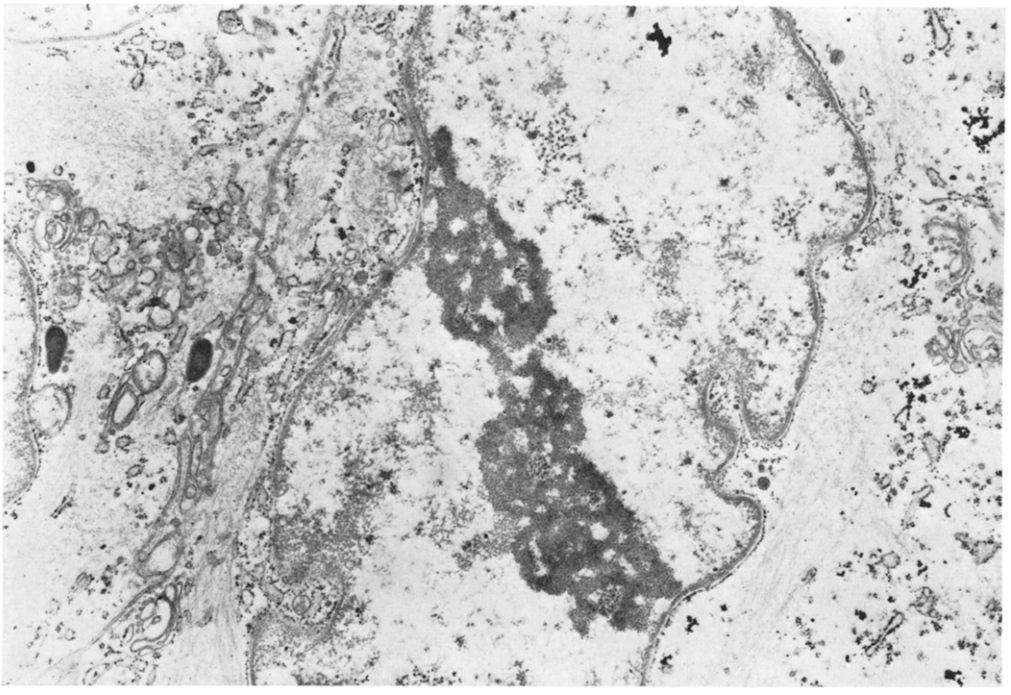


*Fig.* 3(*a*). *T.C.C.* (*Case* 1) *shoring a large mesh-like nucleolus. Well defined nuclear pores and marginated chromatin, and fine structure of the perinuclear fibrillary material,* × 12,020.

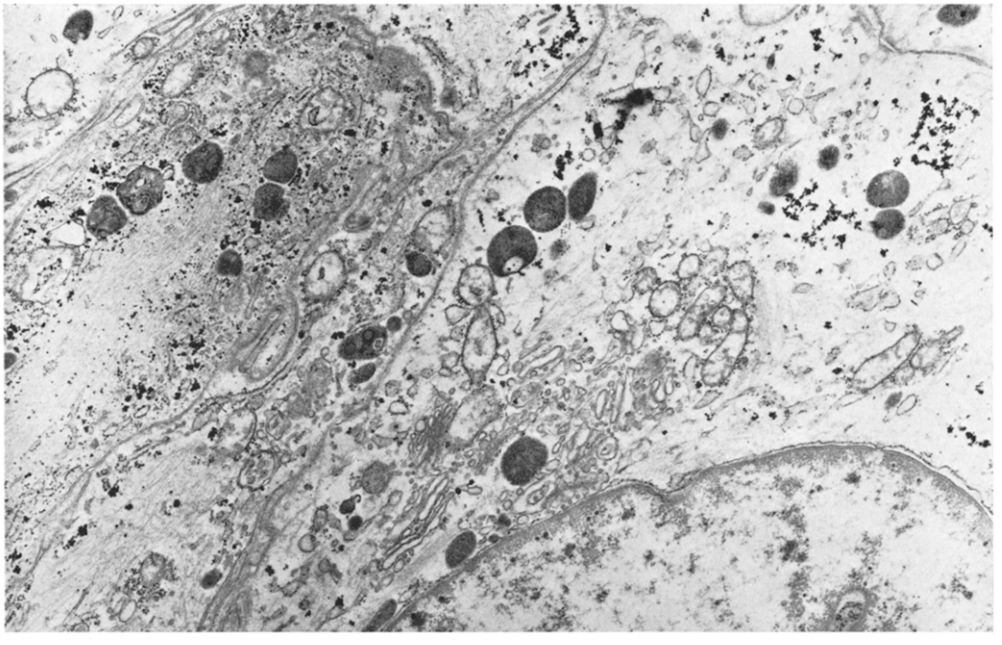


*Fig.* 3(*b*). *T.C.C.* (*Case* 1) *showing Golgi zone and lysosomes, and multi-vesicular bodies,* × 10,000.

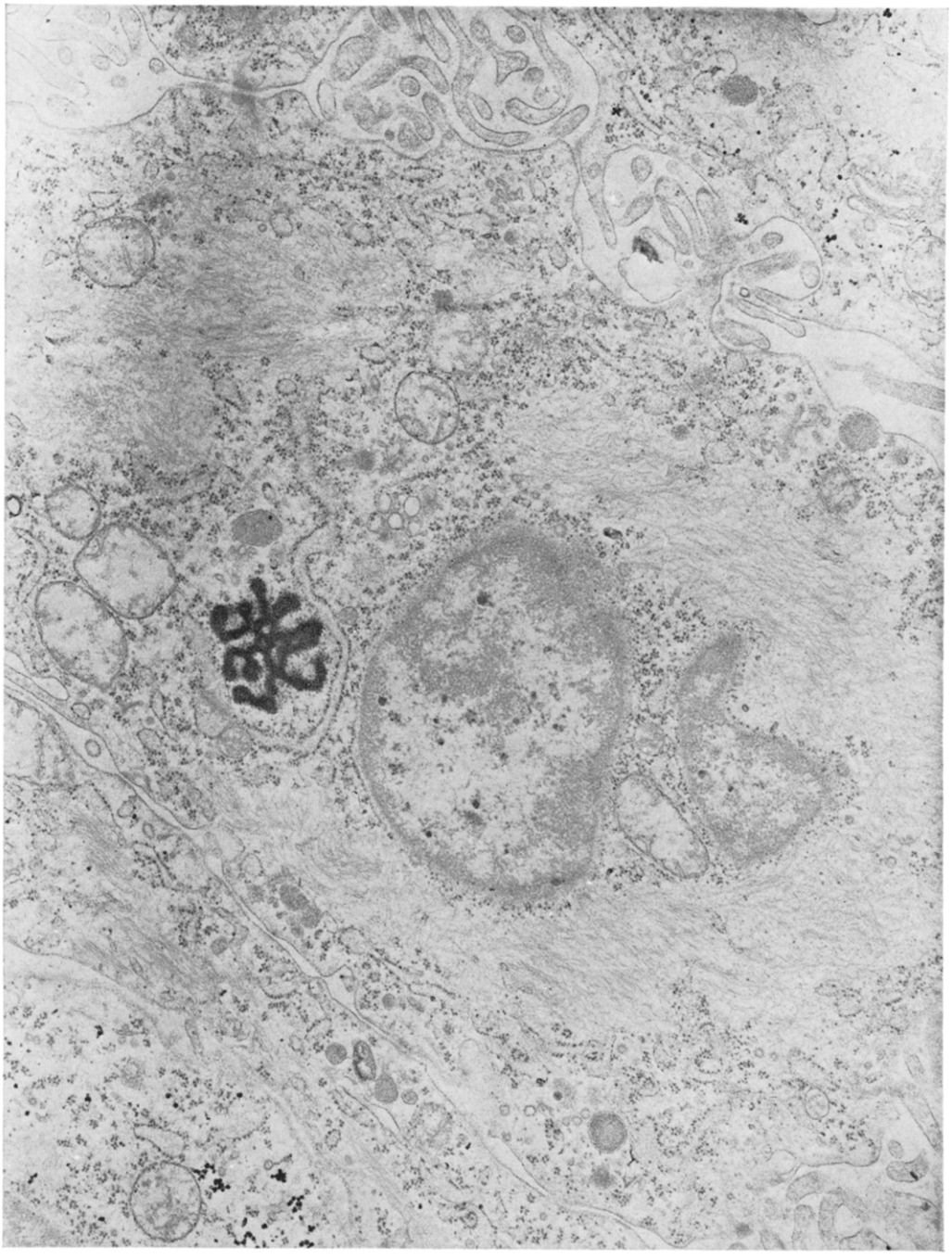


*Fig. 4. T.C.C. (Case 1) showing perinuclear, fibrillary material, high ribosomal density, and a large, organised, ribonuclear body. The cell contacts are variable, either being closely applied or loosely-knit, with interdigating microvilli,* × 18,000.

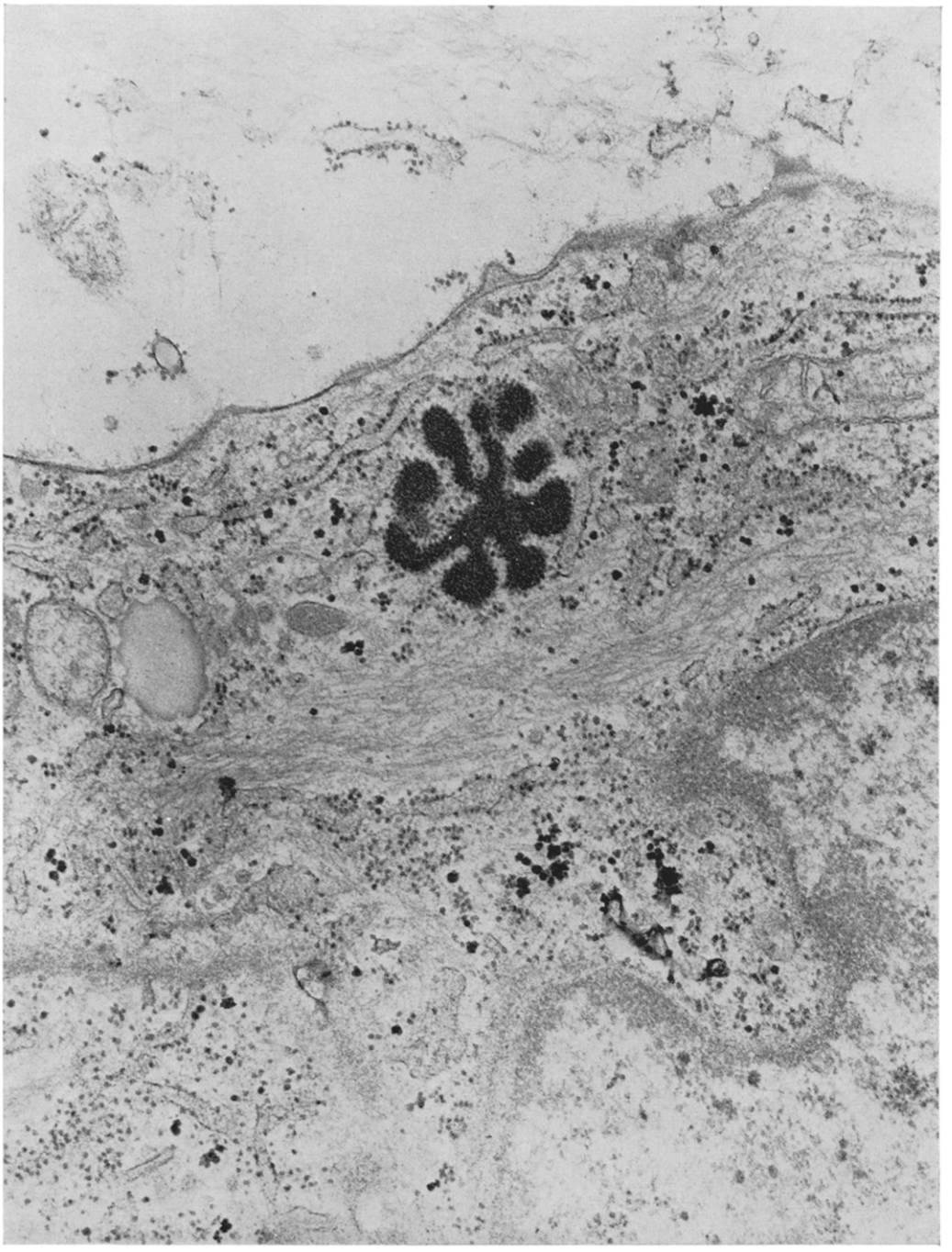
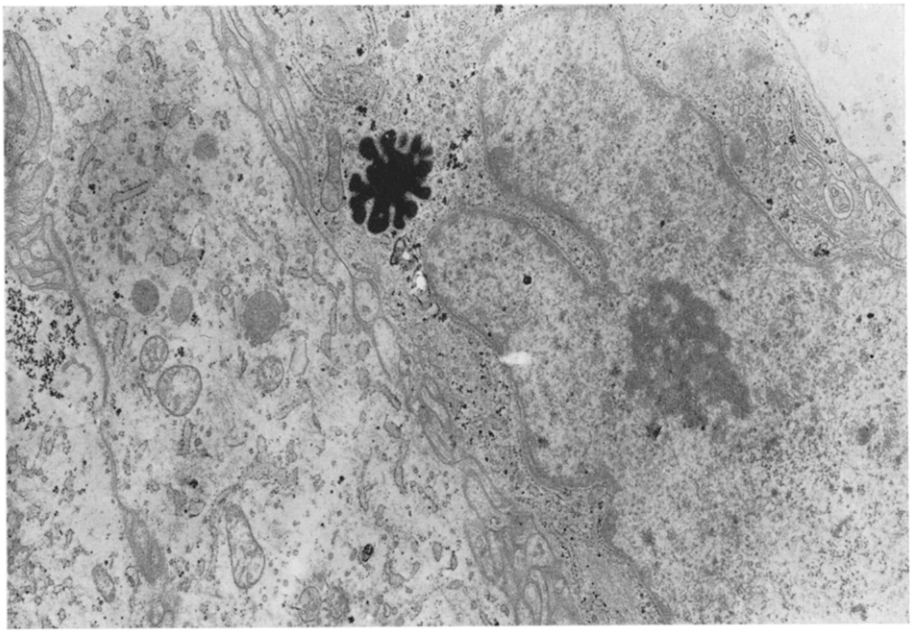
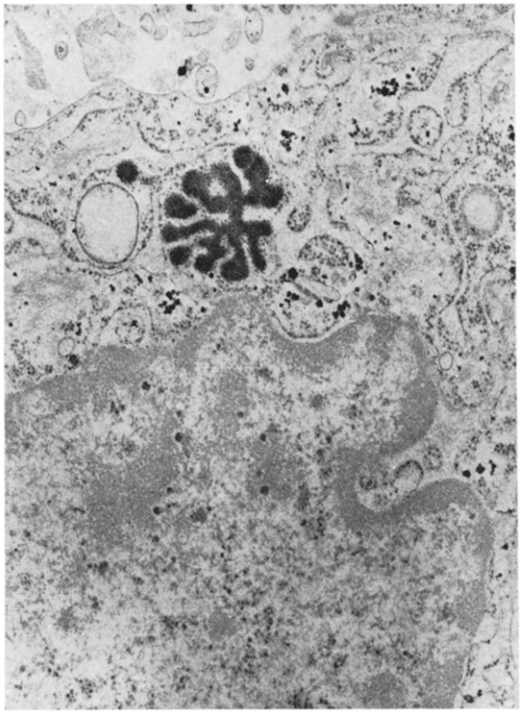


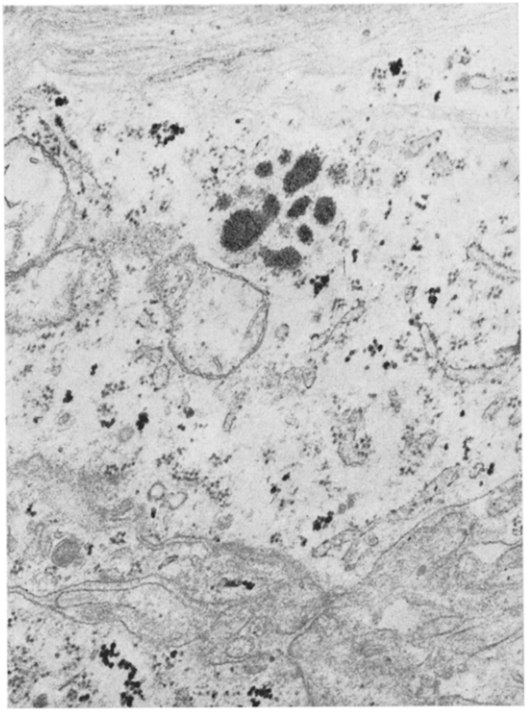
*Fig.* 5. *T.C.C.* (*Case* 1). *Large organized ribonuclear body showing details of ribosomal aggregation. Note distinct difference between morphology of glycogen* (*g*) *and the ribosomes,* × 23,100.



(*a*)



(*b*)



(*c*)

*Fig.* 6. *Examples of ribonuclear bodies in the tumours of Case* 1 *cut at different angles. Note the dense central zone in* (*a*), × 10,000; *a small satellite aggregate away from the main structure* (*b*), × 13,000, *and groups of discrete aggregates* (*c*), × 18,400.

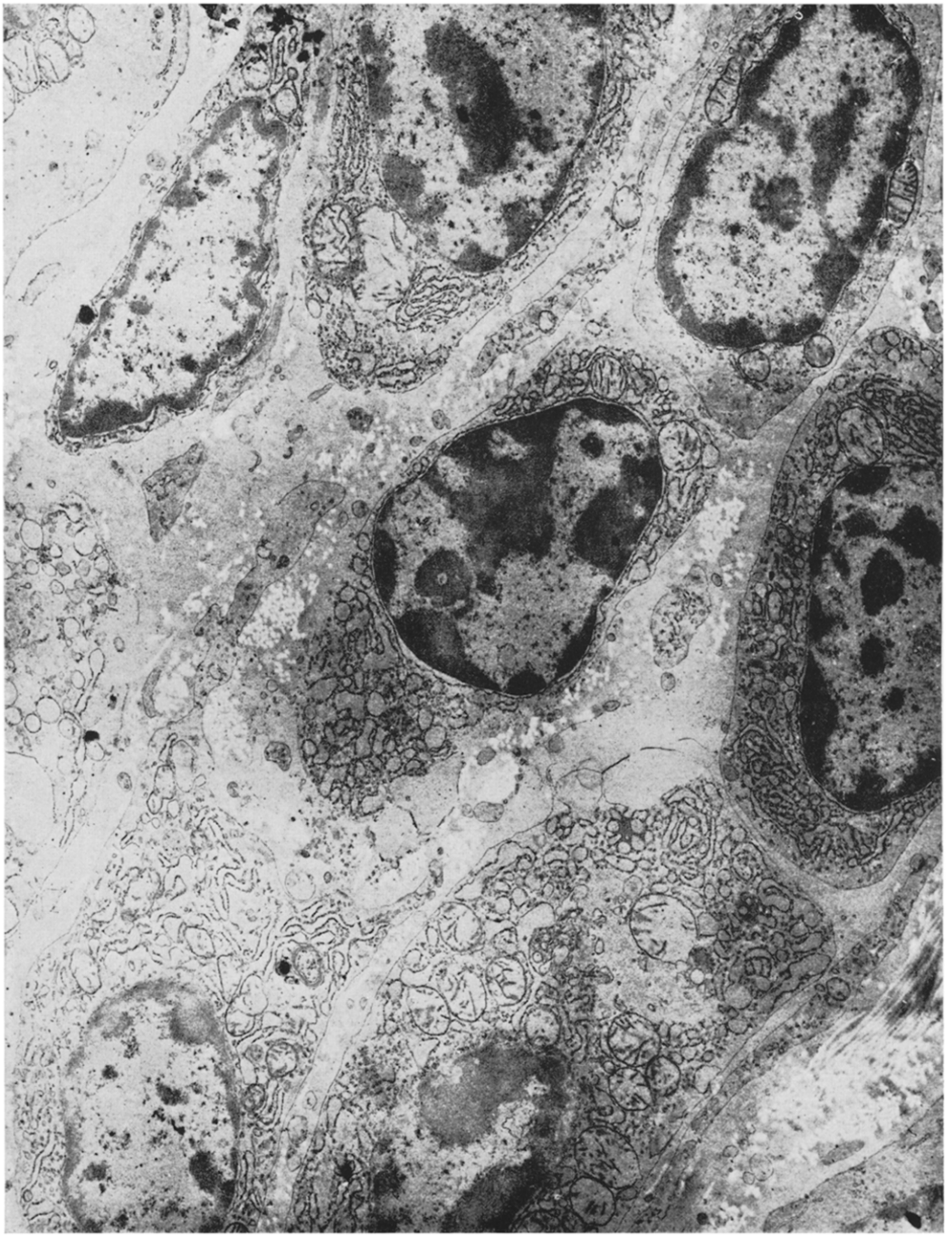


*Fig. 7. T.C.C. (Case 2). A loosely packed area with inter-cellular collagen bands. No microvilli formation. Dense, marginated chromatin. Bizarre mitochondria and endoplasmic reticulum,* × 8620.

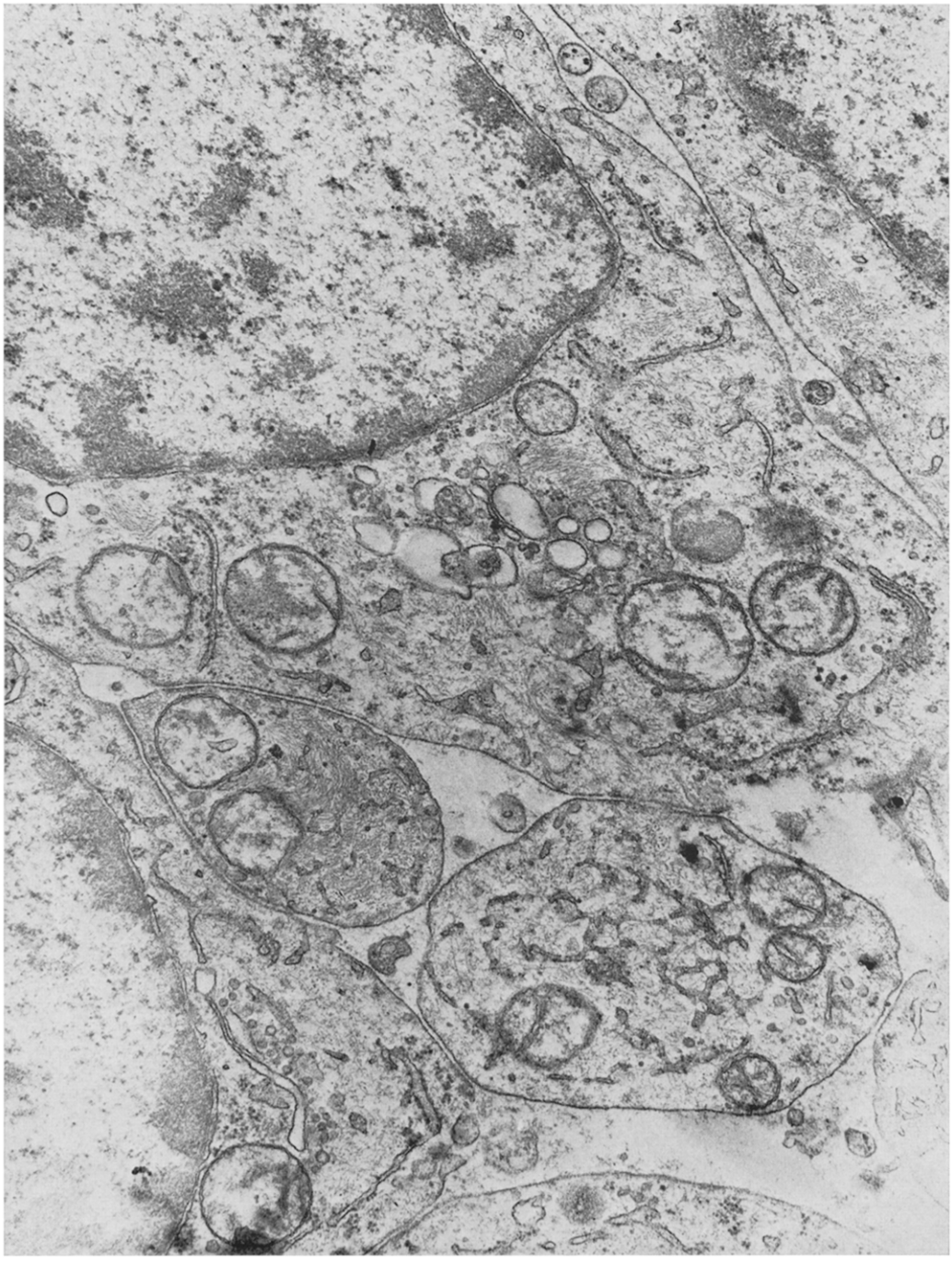


*Fig.* 8. *T.C.C.* (*Case* 2). *Note the diffuse, widely dispersed, cytoplasmic fibrillary material. The endoplasmic reticulum has a paucity of attached ribosomes,* × 16,100.

associations with other cytoplasmic organelles appeared to be random. No comparable structures appear to have been described previously in either normal or neoplastic cells. Desmosomes were occasionally present at the cell contacts and wherever the intercellular space was dilated the cell surfaces bore microvilli.

*Case* 2. The arrangement of cells was more irregular than in either of the tumours of Case 1. The cells tended to be separated in varying degrees, only small areas of the normal intimate apposition being observed (Fig. 7).

In some areas, the wide intercellular spaces contained tracts of collagen fibres. Instead of microvilli, the cell surface either bore larger blunt cytoplasmic protrusions or were almost entirely smooth.

The tumour cell nuclei were ovoid in shape and rarely possessed invaginations of the type observed in normal cells or in the tumour cells of Case 1. Their nuclei were relatively small, while large chromatin aggregates were often present. The cytoplasm contained variable quantities of the granular endoplasmic reticulum. In some cells the abundance of this organelle resembled that found in normal ureteric epithelium cells, varying degrees of ribosomal detachment being present; in others, larger numbers of dilated cisternae were observed, bearing many attached ribosomes. Smooth-surfaced membraneous vesicles were often present, continuous with the granular endoplasmic reticulum. The number of free ribosomes present was similar to that of Case 1 tumour cells.

The Golgi apparatus was invariably atrophic in appearance, consisting of relatively few elements, although the presence of a small number of multivesicular bodies indicated some degree of residual activity. Lysosomes were sparse in these tumour cells.

Cytoplasmic fibrillae were distributed at random in most cells, only rarely were they localized in perinuclear tracts. The quantity present varied considerably from cell to cell (Fig. 8).

The mitochondria of the tumour cells from Case 2 showed great variations in size and form. Frequently they were small and contained few cristae but large dilated forms were also present.

In general, there was considerably more variation in the fine structure of Case 2 tumour cells than was observed among the more highly differentiated cells of the two tumours from Case 1.

## DISCUSSION

The structure of human ureteric epithelium has a general resemblance to that described in laboratory animals [2]. The loose packing of the superficial cell layers, which was a feature of all the specimens, may have been a reflection of their normal state or could have arisen as the result of minor inflammatory or hyperplastic changes that were secondary to the kidney disease that was the underlying reason for the kidney and ureter to be excised. All the control specimens were considered to be within normal limits, as seen in light microscope. A well defined basement membrane, a characteristic feature of transitional epithelium of the bladder in rodents, appeared to be lacking in the ureteric epithelium in man. Unlike the bladder epithelium, the surface cells of the normal ureter did not show any evidence of the highly specialized differentiation, with the formation of fusiform vesicles and frequent polysomes, that has been observed to be a characteristic feature of the surface cells of the bladder epithelium in many species [13–16]. The fusiform vesicles seem to be less pronounced in man [17] but this could be due to loss of surface cells during the taking of biopsies from the bladder.

The tumours had a moderately complex structure and it is likely that they were all growing slowly, as indicated by their pacuity of mitotic figures. Analysis of the DNA content of the tumour cells, reported elsewhere [4] indicated that they were diploid and that only a small proportion of the cells were engaged in DNA synthesis. It is of interest that in Case 1, though the two tumours were uniform in ultrastructure and with respect to their DNA content and proliferative activity, one was far more malignant than the other as judged by its depth of invasion into the ureteric muscle.

The curious ribonuclear bodies observed in the tumours of Case 1 were sufficiently unusual and distinctive to be taken as an indication that, although these tumours were 7 cm apart, they either had a common origin or had been subjected to the same influence, causing these structures to appear. The ribonuclear bodies appear to be formed by the aggregation of ribosomes into branching coral-like structures. Their function is obscure and they do not appear to have been previously reported. The glomerular bodies in the trophoblast cells of rats and mice, described by Toro and Rohlich [18], bear some superficial resemblance to the ribonuclear bodies seen in Case 1. These authors describe glomerular bodies composed of strongly osmophilic threads

forming an irregularly aggregated mass. However, although there were many ribosomes in the vicinity of these structures, they did not appear to form an intrinsic part of the glomerular bodies as they did in the ribonuclear bodies.

In broad terms, the tumour cells showed many features that were a departure from the structure of normal ureteric epithelium; these anomalies were typical of the modifications of the structure of organelles and nuclear organization that are seen in carcinoma and which have been described extensively in the past. However, the tracts of fibrils around the nuclei in Case 1 cells (Fig. 3a) are more specific to T.C.C. They may represent an abnormality of development of the fine fibrils that are an intrinsic part of the structure of transitional cell epithelia being normally most pronounced in the superficial cells of the bladder, though in this site there is no obvious directional orientation. Similar excessive fibrillar formations have been described in transitional cell carcinoma in man and in chemically induced bladder cancers in rats [3]. Loose packing of the epithelium occurred in the normal specimens and in the tumours, and a similar packing was observed in T.C.C. of the bladder [3]. It is well established that the cell turnover rate of normal ureteric epithelium is low and there is evidence to indicate that the tumours were proliferating slowly. This means that, despite their lack of close adhesion, the cells must be relatively stable and are probably anchored to each other by the interlocking of their microvilli.

**Acknowledgements**—We wish to thank Mr. R. E. Williams, of the Department of Urology, Leeds General Infirmary, for providing the specimens, and Dr. C. K. Anderson for his opinion on their histopathology, and Miss Susan Parratt for her technical assistance. This research was supported by a grant from the Yorkshire Council for the British Empire Cancer Campaign for Research.

## SUMMARY

*The ultrastructure of human ureteric epithelium and three ureteric transitional cell carcinoma have been described.*

*Two tumours from the same ureter contained large cytoplasmic bodies (ribonuclear bodies) made of aggregated ribosomes. Abnormality of cytoplasmic fibrils with the formation of perinuclear bands of fibrils was the other characteristic feature in the tumours. The tumours showed several abnormalities of nuclear and organelle structure that are commonly present in malignant cells.*

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# *In Vitro* Effects of Chemical Carcinogens

ANTONIA FLAKS and J. M. HAMILTON
*Department of Experimental Pathology and Cancer Research, School of Medicine, Leeds, Great Britain*

THE polycyclic hydrocarbon 20-methyl cholanthrene (20-MeCh) induces precancerous changes in susceptible pulmonary tissue *in vitro*, which subsequently develop into neoplasia when such tissues are implanted subcutaneously into mice [1, 2]. The capacity of 2-acetyl-aminofluorene (2-AAF), urethane and isoniazid (INH) to induce pulmonary tumours under similar circumstances is investigated in the present report and it is hoped that by the use of this technique it may be possible to determine whether, or not, certain chemical agents are proximate carcinogens for pulmonary tissue.

Wilson, De Eds and Cox [3] reported that 2-AAF produced tumours in a number of organs in rats, including the lungs, while Miller and Miller [4] described the metabolism of and the differing carcinogenic susceptibility of various species to the compound.

Mori and Yasuno [5] and Biancifiori and Ribachi [6] showed that orally administered INH was carcinogenic for mice, while Mori, Yasuno and Matsumoto [7] and Balo [8] obtained a high yield of pulmonary tumours in mice after subcutaneous or intraperitoneal administration of the chemical.

Nettleship, Henshaw and Meyer [9] found that urethane was carcinogenic for the lungs of mice and, since that time, it has been extensively used as a cancer-producing agent and its metabolism studied. The work of Malgren and Saxen [10] and Mirvish [11] indicated that urethane had a direct effect on pulmonary tissue, but the experimental results obtained by Rogers [12], Nery [13] and Boyland and Williams [14] suggested that the chemical had an indirect action on pulmonary tissue and was inactive until metabolised.

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## MATERIAL AND METHODS

Balb/c mice, which are genetically-susceptible to pulmonary neoplasms were used as a source of pulmonary tissue for explants and as hosts for the implants. The donor females were used at one month of age and the hosts were between two and three months old. All mice had been bred in this laboratory by strict brother–sister mating and were fed on Oxoid 41B diet and water *ad libitum*.

Pulmonary explants were about $2 \times 1 \times 1$ mm in size and were maintained in Trowell's chambers in Trowell's medium [15]. The explants were kept *in vitro* for two days in the carcinogen-containing media and for a further day in the normal medium to remove any free carcinogen before implantation into the host animals. The carcinogens examined by this method included 2-AAF (concentration = 10 μg/ml of medium), urethane (concentration = 200 μg/ml of medium) and INH (concentration = 200 μg/ml of medium), while control tissues were cultured in 20-MeCh (concentration = 4 μg/ml of medium) and normal media.

The explants were implanted subcutaneously into the flanks of mice, 24 animals being used for each group. At three-monthly intervals, for one year, six mice were killed and the implants recovered for histopathological examination. The tissues were fixed in 10% formol-saline, embedded in paraffin-wax, sec-

tioned at 5 microns and stained with haematoxylin and eosin.

## RESULTS

The pulmonary tissue implants which had been cultured in the normal medium possessed, on histopathological examination, a normal structural appearance. There was, however, some evidence of perivascular and peribronchial lymphocytic hyperplasia together with occasional bronchial cyst formation. Such findings were common to each stage at which the implants were examined.

Implants which had been cultured in 20-methylcholanthrene medium showed, after three months, lymphocytic hyperplasia which was more prominent in perivascular and peribronchial sites. Well-differentiated adenomata were present. At six months, all implants were extensively affected by adenomatous growth which had displaced the proliferating lymphocytes into a peripheral position around the neoplasms. At nine and twelve months, adenomatous changes had given way to adenocarcinomatous formations which had replaced virtually all of the pulmonary tissue in the implants. The lymphocytic reaction was less evident than in the earlier stages.

Perivascular and peribronchial lymphocytic hyperplasia, together with some bronchial and bronchiolar dilatation, was noted in the implants treated with INH, after three, six, nine and twelve months. There was no evidence of pulmonary neoplastic changes.

2-AAF-treated implants revealed changes similar to those recorded for INH, with the exception that the lymphocytic reaction was more marked and, in one of 24 implants, a small, circumscribed, well-differentiated adenomatous structure was apparent.

Fairly extensive perivascular and peri-bronchial lymphocytic hyperplasia, which spread into surrounding tissues and was associated with bronchial cyst development, was manifested by the implants which had been treated with urethane, but neoplastic changes were entirely absent.

## DISCUSSION

When the activity of different chemical carcinogens was assessed by the presence, or absence, of neoplastic change, the *in vitro* system used in the present studies emphasized the variability of action of such compound on pulmonary tissues. 20-MeCh, which induced adenomatous and adenocarcinomatous lesions, alone proved to be active in a direct manner.

The pulmonary response to urethane was an extensive lymphocytic reaction without adenomatous change and such findings are in accord with those of Rogers [12] and Shabad [16] who were unable to induce pulmonary tumours under similar conditions. It may be considered, therefore, that urethane is inactive in *in vitro* cultures in which it is not metabolized.

INH produced a mild lymphocytic response in and was not adenomogenic for, organ cultures and it is probable that here too, a metabolite is responsible for its indirect pulmonary tumour-inducing ability.

The results obtained with 2-AAF were similar to those described for INH with the exception that one of the 24 implants showed a small adenomatous lesion. The latter, however, was not considered to be of great significance and further, it is known that the compound is mainly metabolised by the liver [4].

The present study has shown that a carcinogenic agent which is capable of direct action on components of pulmonary tissue may be used to induce tumours by *in vitro* methods. However, carcinogenic compounds which may require the participation of whole-body homeostatic mechanisms or have to be metabolised before activation, have proved to be unable to produce neoplastic change in tissue culture preparations. The cause of the lymphocytic proliferation has not been ascertained, but it may arise as a result of the direct effect of the chemical agents upon lymphoid elements or, more probably, it may be a manifestation of a cellular defensive mechanism of the pulmonary tissue. As the host animals showed no abnormalities of their organs and the subcutaneous tissue around their pulmonary implants was normal, it may be reasonable to assume that the hosts did not influence the events.

It is concluded, therefore, that the organ culture method which has been described in the present work, may be used to affirm a chemical agent as a proximate carcinogen for murine pulmonary tissue.

**Acknowledgement**—This investigation was supported by the Yorkshire Council of the British Cancer Campaign for Research.

## SUMMARY

*The present work has been devised to ascertain the possibility of using an* in vitro *technique for the classification of chemical agents into proximate and non-proximate carcinogens.*

*Pulmonary explants, derived from one-month old inbred Balb/c females, were maintained* in vitro *for two days with or without a carcinogen in the medium. They were subsequently implanted subcutaneously into adult mice of the same strain. The implants were examined at three-monthly intervals for one year.*

*The carcinogens examined included* 2-*AAF, urethane and INH, while* 20-*MeCh medium as well as normal medium provided controls.*

20-*MeCh alone proved to be active in a direct manner, judging by the presence of adenomata and adenocarcinomata. On the other hand, the test carcinogens, which require activation* in vivo, *did not induce neoplastic lesions. The culture method may therefore prove to be useful in investigations of the mechanisms of action of chemical carcinogens.*

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# Letter to the Editor

## Preliminary Clinical Screening with Daunorubicin in Lung Cancer

Y. KENIS* and G. BRULÉ†
*Cooperative Group for Clinical Trials in Lung Cancer (EORTC)*‡

The purpose of this trial was to evaluate the effect of daunorubicin§ in lung cancer. It is a screening trial or a type I trial according to the terminology of the EORTC [1, 2] which corresponds approximately to the phase II trials of the National Cancer Chemotherapy Service Center in the United States.

This study included 14 patients, 13 males and 1 female, aged 48–68 years. The diagnosis was epidermoid carcinoma in 9 cases, oat-cell carcinoma in 4 cases and glandular carcinoma in one case. All patients had far-advanced cancer and were not suitable for surgery or radiation therapy, according to the criteria defined in a previous trial of the co-operative group [2].

Daunorubicin was always injected i.v. in a few minutes, with several dose schedules. One patient received 5 daily doses of 20 mg/m2. Eight patients received doses of 35 to 70 mg/m2 at 4–5 days intervals (total dose: 60 to 700 mg/m2). In 2 of these latter, a second and a third series were given after a delay of 2 to 4 weeks. Five patients received 30 to 80 mg/m2 once a week (total dose: 70 to 550 mg/m2).

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*Institut Jules Bordet, Cancer Center of the University of Brussels (Belgium).
†Institut Gustave Roussy, 94 Villejuif (France).
‡The Cooperative Group for Clinical Trials in Lung Cancer (President: Y. Kenis; Secretary: P. Lévy) is a cooperative group of the European Organization for Research on Treatment of Cancer (E.O.R.T.C.).
§Daunorubicin (Daunomycin) was kindly supplied by Farmitalia, Milano (Italy).

### RESULTS

Three cases could not be evaluated since they died within less than 2 weeks after the start of treatment. A radiological improvement was noted in one case. This patient died 4 months after the beginning of the treatment. He received one series of 4 injections and one series of 2 injections of 70 mg/ml each (total dose: 1000 mg). In one case there was a small decrease in size of cervical lymph nodes of less than one month duration, without radiological improvement. The other cases were evaluated as failures.

Three cases were not evaluable (death 2, 7 and 10 days after the start of treatment) for haematologic toxicity. Among the 11 other cases, hemoglobin decreased below 10 g/100 ml in 5 cases, reticulocytes diminished in 9 cases (0 reticulocyte in 7 cases). Serum iron increased in 9 cases. Leukopenia appeared in 10 cases (leucocytes 3000/mm$^3$ in 1 case; 2700 in 1 case; 1200 in 1 case; less than 1000 in 7 cases). Thrombocytopenia (platelets less than 100,000/mm$^3$) was observed in 3 cases.

Cardiac toxicity was studied by frequent determinations of several enzymes, among them creatin phosphokinase (CPK), and weekly electrocardiograms. Three patients died within the 10 first days of treatment. Death was attributed to complications directly related to the lung cancer. In the other patients, CPK increased to abnormal values during the treatment in 8 cases. Abnormal values of lactic dehydrogenase were present in 4 of these cases. There were no significant neither constant alterations of the e.c.g. Signs and

symptoms of cardiac failure were observed in one single case, 8 days after the end of the second series of injections of daunorubicin (total dose: 800 mg).

A perforated ulcus occurred in one patient after the second dose of daunorubicin. This patient died 4 days after a gastroraphy, with acute respiratory insufficiency.

## CONCLUSION

This preliminary trial was discontinued when there was a clear-cut evidence of an objective response in one case [1]. It was not considered necessary to treat a larger number of patients because the hematologic toxicity was found to be too severe. With the dose schedule utilized in this trial the maximum tolerated dose was reached in each case but one. The therapeutic results were considered too insignificant to continue the trial.

It is concluded that daunorubicin at these doses and with this regime had some effectivness in lung cancer but that the cardiac and hematologic toxicities were too important to permit an adequate treatment. Consequently daunorubicin was discarded in the routine treatment of far-advanced lung cancer.

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# Letter to the Editor

## Fibrinolysis, Thromboplastin Activity and Localization of Radioiodinated Fibrinogen in Experimental Tumors

P. HILGARD and V. HIEMEYER

*Department of Hematology and Blood Coagulation Research, Center of Internal Medicine and Pediatrics, University of Ulm, Germany*

SOME experimental animal tumors and human tumors take up radioiodinated fibrinogen [1–4]. This property has been used by some investigators for diagnostic and therapeutic trials [2, 5]. Several authors [2, 6] were able to show an interference of anticoagulants with the uptake of fibrinogen by the tumor tissue. This fact suggested that the coagulation mechanism plays an important role in localization of fibrinogen in neoplasms.

Our own investigations have demonstrated that the Yoshida sarcoma and the Ehrlich carcinoma accumulated radioiodinated heterologous fibrinogen to a high degree, whereas the isologous DS-carcino-sarcoma [7] and the authochtonous benzpyrene sarcoma showed very little uptake of the injected labeled fibrinogen. Accordingly no presentation in the dot scan was found in the latter tumors. Even treatment with very high doses of E-amino caproic acid (4g/animal), beginning 4 days before the fibrinogen injection had no significant influence on the uptake of radioactivity by these tumors. These data suggested that fibrinolysis was not responsible for the lack of uptake. To confirm this, investigations of the fibrinolytic system of benzpyrene sarcoma-bearing rats were carried out.

Plasminogen and plasmin were determined according to the slightly modified caseinolytic method of Alkjaersig *et al.* [8]. Plasma was activated with human urokinase. The antiplasmin level was estimated by the proteolysis inhibiting capacity of plasma against porcine plasmin in a caseinolytic assay.

*Table* 1. *Mean plasminogen and antiplasmin level in* 30 *benzpyrene sarcoma bearing rats and in* 30 *normal healthy rats.*

| | Plasminogen U/ml | | Antiplasmin U/ml | |
|---|---|---|---|---|
| | mean | S.D. | mean | S.D. |
| Normal rats | 227·4 | 26·2 | 70·4 | 9·3 |
| Tumor rats | 126 | 33·1 | 88·6 | 8·7 |

Table 1 shows the mean values and the standard deviation of the plasminogen and antiplasmin levels in 30 tumor-bearing and 30 normal-healthy rats (Wistar strain). It is evident that the plasminogen level in tumor animals is significantly decreased, whereas the anti-plasmin level is clearly elevated compared to the control animals. Free proteolytic activity —suggesting plasmin activity—was found in 70% of the normal and only in 30% of the tumor animals. These findings show that the whole fibrinolytic potential in benzpyrene sarcoma-bearing rats is considerably decreased. This may be an explanation why a fibrinolysis

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inhibitor cannot affect significantly the uptake of radioiodinated fibrinogen by this tumor.

Further investigations were designed to determine the thromboplastin activity of the tumors used in our scan experiments according to Quick's one stage method [9]. One gram of tissue was homogenated with 2 ml saline. After centrifugation, 0·1 ml of the supernatant and 0·1 ml m/40 $CaCl_2$ solution were added to 0·1 ml of normal citrated rat plasma. The clotting time was measured in a water bath at 37°C.

*Table 2. Thromboplastin activity of tissue extracts from various experimental animal tumors*

| | Thromboplastin activity sec. | |
|---|---|---|
| | mean | S.D. |
| Yoshida sarcoma | 10·3 | 1·2 |
| Ehrlich carcinoma | 19·5 | 1·2 |
| DS-carcino-sarcoma | 25·1 | 1·1 |
| Benzpyrene sarcoma | 28·4 | 1·3 |
| Standard thromboplastin | 11·5 | 1·0 |

Table 2 shows that the thromboplastin activity of the Yoshida sarcoma was greater than that of a standard thromboplastin solution (Roche). There was some thromboplastin activity in the Ehrlich carcinoma while the DS-carcino-sarcoma and the benzpyrene sarcoma had much less thromboplastin activity. It thus turned out that those tumors which had a positive dot scan after injection of $^{131}$I-fibrinogen had a considerably stronger thromboplastin activity than the remaining tumors which had negative dot scans.

In summary, in the tumors we investigated, thromboplastin activity correlates well with radioiodinated fibrinogen uptake and fibrinolysis has little influence on the uptake of labeled fibrinogen. In order to make the injection of $^{131}$I-fibrinogen a useful instrument for diagnosis of malignant tumors in men, parameters should be found to determine whether a tumor has enough thromboplastin activity to cause fibrin deposition which can be recognised in the dot scan. Studies of coagulation factors in cancer patients do not demonstrate any cancer specific alteration [10, 11]. In our opinion, only systematic investigations of the thromboplastin activity of a great number of human neoplasms can determine whether dot scans after $^{131}$I-fibrinogen injection are of any help in cancer diagnosis.

**Acknowledgements**—This work was supported by grant of the "Landesverband Württemberg zur Erforschung und Bekämpfung des Krebses".

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*Europ. J. Cancer* Vol. 6, p. 159. Pergamon Press 1970. Printed in Great Britain

# Announcement

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**LA REVUE FRANÇAISE D'ETUDES CLINIQUES ET BIOLOGIQUES**

*devient*

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Les *Notes originales*, courts articles de 4 à 6 pages dactylographiées, seront publiées dans les *deux mois* à partir de leur acceptation définitive.

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Original papers
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# Studies on the Cellular and Molecular Mechanisms of Hydrocarbon Carcinogenesis

CHARLES HEIDELBERGER*

*McArdle Laboratory for Cancer Research, The Medical School, University of Wisconsin, Madison, Wisconsin, 53706, U.S.A.*

I AM DEEPLY appreciative and cognizant of the honor of being named the first Walter I. Hubert lecturer, which gives me the privilege of addressing the 10th annual meeting of the British Association for Cancer Research. It is with some temerity that I speak about carcinogenic hydrocarbons in Britain, the land where they were first postulated, isolated, and tested; and where in the very air of this city of Manchester they make a lasting abode.

In the 194 years since Percival Pott's [1] keen observations and brilliant deductive reasoning inaugurated our field of cancer research in general, and chemical carcinogenesis in particular, England has been the setting for the major advances in our long and painful struggle to understand the cellular and molecular mechanisms of hydrocarbon carcinogenesis. In this setting, I feel abashed and redundant, as should one who carries coals to Newcastle or benzpyrene to Manchester.

It was the Scottish organic chemist, J. W. Cook with his colleagues Hewett, Hieger and Mayneord [2] who followed the lines of the 1,2-benzanthracene fluorescence spectrum seen in coal tar, and synthesized 1,2,5,6-dibenzanthracene, the first pure chemical compound to induce cancer. And the same team in 1933 isolated a few milligrams of pure crystals from several tons of coal tar, which they showed by synthesis to be 3,4-benzpyrene [3], a compound immortalized in the lungs of every cigarette smoker and residents of Manchester, Birmingham, Leeds and Los Angeles. The chemists were supported and inspired by the biological insight and testing procedures devised by Sir Ernest Kennaway, and the team of Cook, Kennaway, and their colleagues wrote a brilliant chapter in this as yet unfinished saga.

More recently this great British tradition in carcinogenesis has been continued by Sir Alexander Haddow, who first discovered the growth-inhibitory effects of carcinogenic hydrocarbons [4], and whose wisdom, foresight, and administrative skill have nurtured and fostered generations of creative scientists in a unique Institute. One of his colleagues is Boyland, whose studies on hydrocarbon metabolism conducted with imagination and patience have provided most of the fundamental information that we have on this subject [5,6]. Berenblum, when he was at Oxford, discovered co-carcinogenesis and initiation and promotion [7]. More recently, the British pre-eminence, in this field has been maintained by Brookes and Lawley with their studies of the binding of carcinogenic hydrocarbons to DNA, a logical continuation of their research on the reactions of alkylating agents with DNA [8,9]. And finally I would like to pay tribute to the biological skill of Ilse Lasnitzki, whose work on the effects of carcinogenic hydrocarbons on organ cultures of mouse prostate [10] was the inspiration for and whose patient

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counsel and tutelage made possible our more recent efforts.

My own interest in hydrocarbon carcinogenesis was stimulated while I was a graduate student of L. F. Fieser, who was an American counterpart of J. W. Cook in that he synthesized many carcinogenic hydrocarbons, such as methylcholanthrene. However, I was not working on that problem, which was shelved in favor of wartime antimalarial research. My entrance into the field occurred 23 years ago, when working at the Radiation Laboratory of the University of California at Berkeley I accepted the challenge to build the first millicurie of carbon-14 ever used for the synthesis of a compound more complicated than a 3-carbon acid, into 1,2,5,6-dibenzanthracene [11]. And I have been working with it ever since — but not with the very same millicurie! The first effort with the labeled hydrocarbon was to study its tissue distribution and metabolic degradation following various routes of administration to mice [12, 13]. After moving to Wisconsin in 1948 I continued working in this field and correlated the rates of disappearance of 3 hydrocarbons from the site of subcutaneous injection with their carcinogenic activities [14], showed metabolic ring cleavage of 1,2,5,6-DBA [15], and identified some new quinonoid metabolites of the same compound [16]. We also synthesized all the possible mono- and dihydroxy derivatives of 1,2,5,6-DBA [17], thereby showing that the hitherto long unidentified rabbit metabolite was 2′,6′-dihydroxydibenzanthracene.

However, most of the above mentioned work appeared to be concerned with metabolic degradation and detoxication, rather than with carcinogenesis itself. Since the time of Ehrlich it has been axiomatic that in order for a drug to exert a pharmacological action it must interact with some cellular receptor. In 1951, E. C. Miller [18] demonstrated by means of fluorescence techniques that benzpyrene was firmly bound to the proteins of mouse skin, and we have continued and extended this finding, using at first $^{14}C$ and later tritiated hydrocarbons as the analytical tool. At first we developed methods of protein isolation from mouse skin after a single topical application (which initiates carcinogenesis when followed by croton oil [7]) of 1,2,5,6-DBA, and demonstrated that it was bound covalently to the soluble and insoluble proteins [19]. Then we found a quantitative correlation between the carcinogenic activities of a series of hydrocarbons and the extent to which they were bound to the soluble proteins, with the exception of 1,2,3,4-dibenzanthracene, which is essentially non-carcinogenic, and was bound to a high extent [20]. This exception turned out to be fortuitous, for it gave us a probe to distinguish binding that is related to carcinogenesis from non-specific binding. At the same time we were working on the structure of the protein-bound dibenzanthracene, and identified one of the hydrolysis products as being a dicarboxylic acid resulting from ring cleavage at the "K" region [21, 22]. Unfortunately, on repetition of this work by Dr. A. R. Somerville of Manchester and others in my laboratory using more sophisticated chromatographic methods, this identification was not confirmed. Also, with Dr. Somerville we disproved the suggestion of zero-time binding [23].

We then started to fractionate the soluble proteins, looking for a fraction to which the carcinogenic 1,2,5,6-DBA was bound and to which the non-carcinogenic 1,2,3,4-DBA was not bound [24]. Abell and I found that on starch-gel electrophoresis of the soluble proteins of mouse skin, following topical application *in vivo* of various hydrocarbons, there was a band to which the carcinogenic hydrocarbons were bound to a much greater extent than the non-carcinogenic ones [25]. A plot of the radioactivity of methylcholanthrene vs. 1,2,3,4-DBA is shown in Fig. 1. When the results of various hydrocarbons were compared, as shown in Table 1, there was an excellent quantitative correlation between the binding of

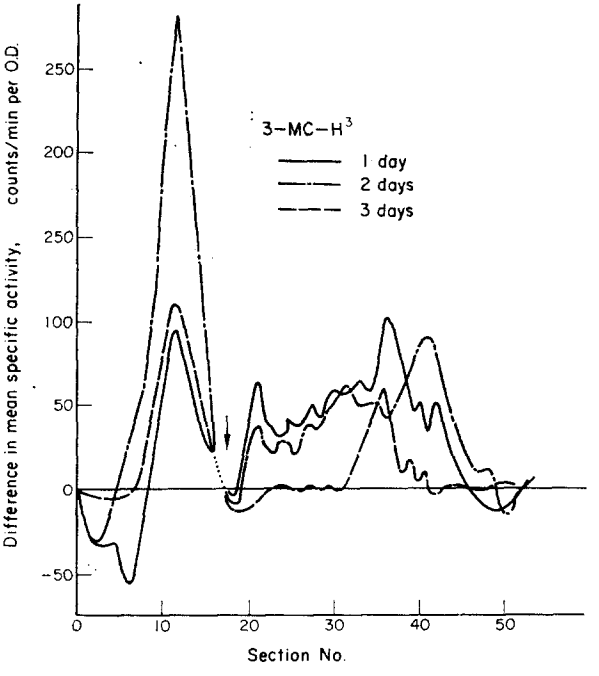


*Fig. 1. Difference curves of specific activities of mouse-skin proteins on starch-gel electrophoresis after applications of MCA and 1,2,3,4-DBA* [25].

*Table* 1. *Specific activity of protein fraction in starch-gel**

| Tritiated hydrocarbon | Carcinogenic activity | Specific activity cpm/O.D. | Time |
|---|---|---|---|
| Methylcholanthrene | +++ | 423 | 2 days |
| 9,10-Dimethyl-1,2,5,6-DBA | +++ | 278 | 2 days |
| 9,10-Dimethyl-1,2-benzanthracene | +++ | 189 | 1 day |
| 3,4-Benzpyrene | +++ | 280 | 4 hr |
| 1,2,5,6-DBA | ++ | 180 | 2 days |
| 10-Methyl-1,2-benzanthracene | ++ | 160 | 2 days |
| 4-Fluoro-10-methyl-benzanthracene | ++ | 75 | 2 days |
| 4-Methyl-1,2-benzanthracene | ± | 80 | 2 days |
| Anthranthrene | — | 22 | 1 day |
| 1,2,3,4-DBA | — | 25 | 1 day |
| 3-Fluoro-10-methyl-1,2-benzanthracene | — | 6 | 1 day |
| 3-Methyl-1,2-benzanthracene | — | 6 | 1 day |

*[25].

hydrocarbons to this fraction and their carcinogenic activities. Moreover, this fraction was absent from tumors induced by the hydrocarbons. In 1947 the Millers [26] discovered that the carcinogenic aminoazo dyes were covalently bound to rat liver proteins after feeding. Electrophoretic fractionation of this protein has been carried out by Sorof *et al.* [27], and we showed that this protein bore an electrophoretic resemblance to the protein that we were working with in mouse skin [25]. This aminoazo dye protein conjugate has been extensively purified in this country by Ketterer *et al* [28]. We have undertaken further purification of the mouse skin protein to which the carcinogenic hydrocarbons are specifically bound, and considerable, but frustratingly slow, progress has been made. Sorof *et al.* [29] have reported that a protein migrating electrophoretically in the same region as the azo dye-bound protein from rat liver, and which

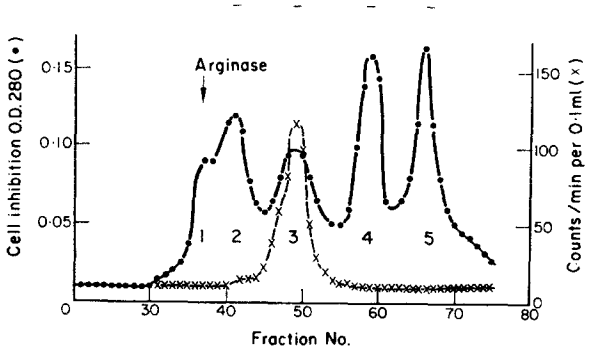


*Fig.* 2. *Sephadex G-100 fractionation of partially purified mouse-skin proteins, together with arginase and cell growth inhibition results* [30].

inhibits the growth of cells in culture, is actually liver arginase. This prompted us to find out whether the hydrocarbon-bound protein from mouse skin is arginase. As shown in Fig. 2, fractionation of partially purified protein on Sephadex G100 completely separated the arginase activity from the radioactive protein; the arginase-containing peak inhibited the growth of HeLa and L-5178Y cells in culture, but none of the other fractions had inhibitory activity [30]. We are continuing work in order to purify, isolate, characterize, and hopefully to determine the role of this protein (if any) in the carcinogenic process.

While the protein binding work was going on, we also investigated the possible binding *in vivo* of 1,2,5,6-DBA to mouse skin DNA. Using the crude methods of isolation that were available in 1961, Davenport and I [31] isolated degraded DNA, which contained radioactivity, and this label was released from a dialysis bag in the presence of DNAse and diesterase (Fig. 3). Therefore, this was evidence for the firm binding of the carcinogen to DNA. Using more elegant methods of extracting DNA from mouse skin with phenol, Brookes and Lawley [8] confirmed and extended this observation to other hydrocarbons, and obtained a correlation between the binding of hydrocarbons to mouse skin DNA and the Iball index of their carcinogenic activities [9]. At the same time we were continuing and also extending this work by measuring the *in vivo* binding of four hydrocarbons to the DNA isolated from the epithelial cells of mouse skin, prepared as worked out by

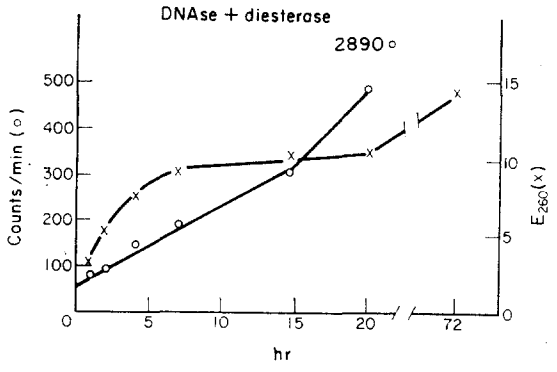


*Fig. 3. The release of optical density and radioactivity from dialysis of mouse-skin DNA treated with DNAse and diesterase* [31].

Giovanella and myself [32]. The specific activities of four hydrocarbons bound to DNA are shown in Fig. 4, and it is seen that 1,2,3,4-DBA is bound to a greater extent to DNA than is 1,2,5,6-DBA [33]. Consequently, we are not as impressed with the correlation between carcinogenesis and binding to DNA as is Brookes. However, instead of fighting, we joined. Peter Brookes spent a year in my laboratory as an Eleanor Roosevelt Fellow of the UICC, and the modest fruits of this collaboration were recently published [34] evidence being obtained, as indicated in Fig. 5, that DMBA bound to mouse fibroblast DNA in culture is attached to nucleotides and to the free bases. Dr. Brookes is continuing this work to elucidate the chemistry of the bound hydrocarbons.

In almost every case that has been properly studied, it has now been shown that chemical carcinogens are covalently bound to DNA, RNA and protein of the target cells. To prove which, if any, of these macromolecules is the critical target of carcinogenesis, has as yet been beyond the capabilities of all investigators

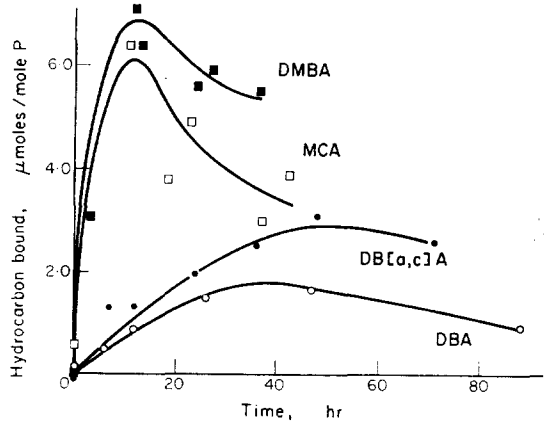


*Fig. 4. Specific activities of the DNA isolated from the epithelial cells of mouse-skin following application of four hydrocarbons* [33].

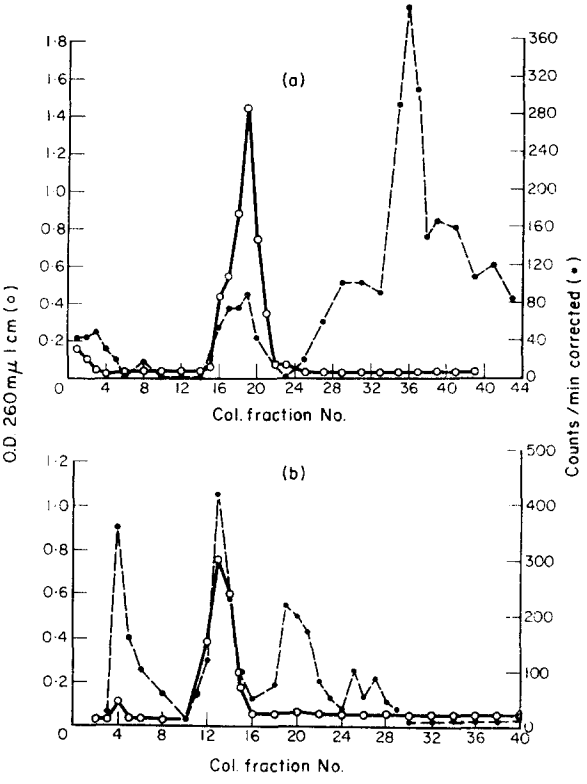


*Fig. 5. DEAE cellulose chromatography of DNA of mouse embryo fibroblasts grown in the presence of $^3$H-DMBA. A represents DNAse* (1 *hr*) *and diesterase* (25 *hr*). *B represents DNAse* (1 *hr*) *and diesterase* (42 *hr*) [34].

in the field. However, since the hydrocarbons are not chemically reactive, it has become clear that they must be metabolically activated to a form that reacts chemically with DNA, RNA and proteins. The chemistry of the metabolic activation of acetylaminofluorene and the aminoazo dyes has been brilliantly elucidated by the Millers [35]. Space does not permit describing this fundamental work. Suffice it to say that they have demonstrated that N-hydroxylation of the amines takes place, followed by the formation of chemically reactive esters that attack nucleophilic sites, the 8-carbon of guanine in DNA, RNA, and the sulfur atom of methionine in proteins. The reaction of these esters with transforming DNA produces mutations [36], and treatment of bacteriophage T4 with the same compounds also produces mutations (Corbett, Dove and Heidelberger, in preparation). The structures of the azo dyes bound to proteins has recently been determined [37]. It may be postulated that the carcinogenic hydrocarbons are also metabolically activated to electrophilic reagents and Dipple, Lawley and Brookes have recently set forth a molecular orbital theory as to how this might happen [38]. In our laboratory we have resumed a study to determine the

structures of the protein-bound derivatives of dibenzanthracene in order to infer the structure of the reactive metabolites. A major advance in this direction has been recently made by Grover and Sims [39] who have found that when various hydrocarbons are allowed to react with the liver microsomal drug-metabolizing enzymes, they bind to added DNA. Similar work has been done by Gelboin [40].

Depending on whether the binding to DNA or to RNA or protein is most closely involved in hydrocarbon carcinogenesis, we can imagine two general molecular mechanisms; (1) somatic mutation, resulting from the binding to DNA and alteration of its structure; or (2) modification of gene expression, which could occur in several ways including derepressions. Although it was difficult to see how the 2nd mechanism could lead to perpetuated changes, Pitot and I [41], purely as an intellectual game postulated that carcinogenesis could result from the alteration of a Jacob and Monod metabolic regulatory circuit, as shown in Fig. 6. This

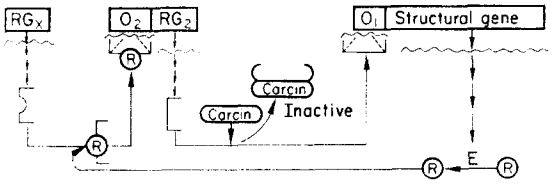


*Fig. 6. Modified Jacob–Monod regulatory circuit to explain carcinogenesis* [41].

topic I have discussed more fully [42, 43]. It must be clearly stated that at present the issue between the mutational and non-mutational mechanism has not been settled; each has its enthusiastic and vocal advocates.

However, after years of glib thoughts, conversations, and lectures about molecular mechanisms of chemical carcinogenesis, the sobering thought gradually came to me that before we can understand molecular mechanisms, we must know something about the cellular mechanisms. Three alternatives present themselves: (1) the chemical transforms a normal cell into a cancer cell; (2) the carcinogen selects for pre-existing cancer cells, as postulated by Prehn [44]; or (3) the hydrocarbon activates a latent oncogenic virus. Although most workers in the field (including myself) have presupposed mechanism 1, until very recently there has been no decisive evidence for or against any of these mutually exclusive mechanisms. Obviously, one would design very different sorts of biochemical experiments depending on which of these cellular mechanisms obtained. After some thought, it seemed to me that this matter could only be settled by experiments on carcinogenesis *in vitro*, and so I set out to develop a suitable system.

At the time this decision was made, rather dazzling techniques had been developed by virologists for quantitating the various phenomena of virus-cellular interactions, and I hoped that somewhat similar methods might be successfully applied to chemical carcinogenesis *in vitro*. But where to start? Nobody at that time had seriously attempted studies of this sort. But one lead had been uncovered by Lasnitzki, who had shown that carcinogenic hydrocarbons produced morphological changes suggestive of neoplasia in organ cultures of mouse prostates [10]. She kindly accepted a neophyte biologist into her laboratory at the Strangeways in Cambridge, and in 1962 I spent a delightful sabbatical, learned much from her, but unfortunately gave little in return.

Back in Madison, Dr. Manuela Röller and I set to work with this system adapted to liquid media and using the prostates from inbred C3H mice. In general, the controls maintained their differentiation (Plate 1), and the treated organ cultures exhibited massive hyperplasia, squamous metaplasia (Plate 2), and occasionally invasion through the basement membrane. Some of these treated cultures were read by pathologists as being malignant. However, on injection of 872 of such cultures into as many mice under a variety of conditions, no tumors were produced [45]. Therefore, in spite of the profound morphological alterations, carcinogenesis *in vitro* in this system was not accomplished.

At this time, Dr. Thomas Iype from India joined my group and we treated the organ cultures from the hydrocarbon experiments with pronase, and then grew them in cell culture. These formed permanent lines, were resistant to the toxicity of hydrocarbons, and produced malignant transplantable tumors in C3H mice. Most of these were sarcomas, but there were a few carcinomas. Unfortunately, we could not obtain cell lines from the organ cultures that were not treated with hydrocarbons, so we could not completely rule out the possibility that spontaneous malignant transformation, that is almost ubiquitous in mouse cells [46], might have accounted for our results [47, 48].

Dr. T. T. Chen worked in my laboratory on the problem of growing cells from the non-carcinogen treated organ cultures, and by using some unorthodox techniques he finally succeeded. The growth rate of the cells during

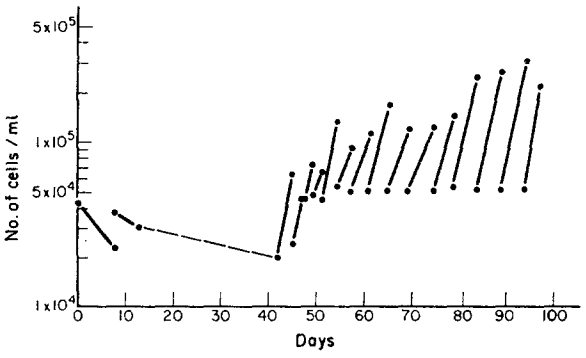


*Fig. 7. Beginning of B1 prostate cell culture* [48].

the establishment of the culture is shown in Fig. 7. It took some time for these cells to start growing, and the life history of the culture is shown in Fig. 8 [49]. This cell line has been maintained in culture for a prolonged period of time, and spontaneous malignant transformation did not occur until 570 days in culture. This cell line until that time did not produce any tumors with $10^6$–$10^7$ cells inoculated subcutaneously into irradiated C3H mice. The cells were aneuploid almost from the beginning,

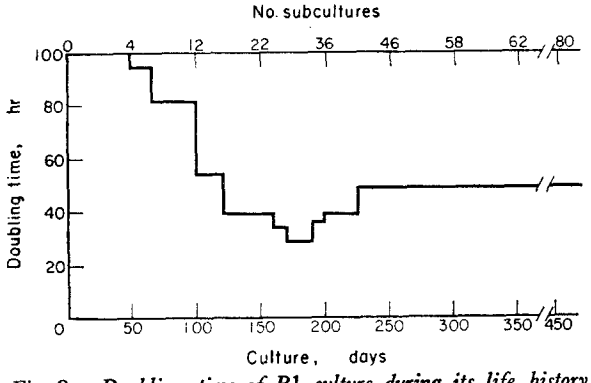


*Fig. 8. Doubling time of B1 culture during its life history* [49].

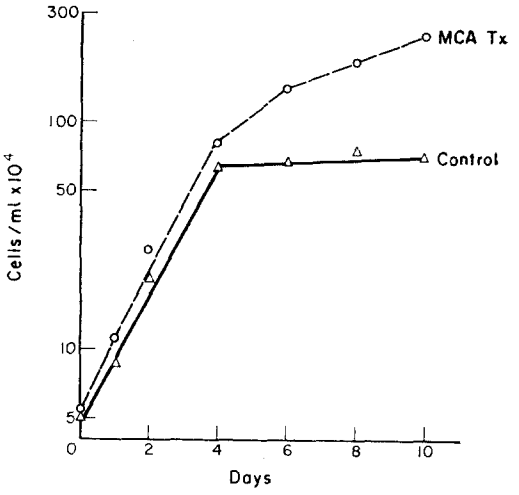


*Fig. 9. Growth characteristics of control prostate cells and those transformed by MCA* [50].

and this cell line (although several others were established and behaved comparably) has been the source of much of the work I now report.

One of the major characteristics of these cells is that they reach a saturation density once a monolayer is formed, as shown in Fig. 9. However, it was discovered that if methylcholanthrene (MCA) were added, the cells continued to grow after a monolayer was attained and piled up on top of each other to give a characteristic random, criss-cross orientation, as also shown quantitatively in Fig. 9 [50]. It was soon found (Table 2) that the property of forming piled-up colonies coincided with the ability to give tumors in unconditioned C3H mice. In fact, 1000 cells gave a 100% incidence of fibrosarcomas when injected subcutaneously, whereas $10^6$ cells derived from 0·5% DMSO (the solvent for the hydrocarbons) treatment gave no tumors in irradiated mice. Therefore,

*Table 2. Tumor formation in adult C3H male mice after inoculation subcutaneously of prostate cells**

| Treatment | Days in culture after isolation of colonies | No. cells inoculated | Days after inoculation | Palpable tumors |
|---|---|---|---|---|
| Control | 3 | $1\times10^4$ | 150 | 0/4 X-ray |
| MCA | 3 | $1\times10^4$ | 21 | 4/4 |
| Control | 7 | $1\times10^6$ | 146 | 0/4 X-ray |
| MCA | 7 | $1\times10^6$ | 14 | 6/6 |
| Control | 11 | $1{\cdot}3\times10^6$ | 142 | 0/4 X-ray |
| MCA | 11 | $1\times10^3$ | 28 | 2/2 |
| MCA | 11 | $1\times10^4$ | 21 | 2/2 |
| MCA | 11 | $1\times10^5$ | 14 | 2/2 |
| MCA | 11 | $1\times10^6$ | 14 | 2/2 |

*Chen and Heidelberger [50].

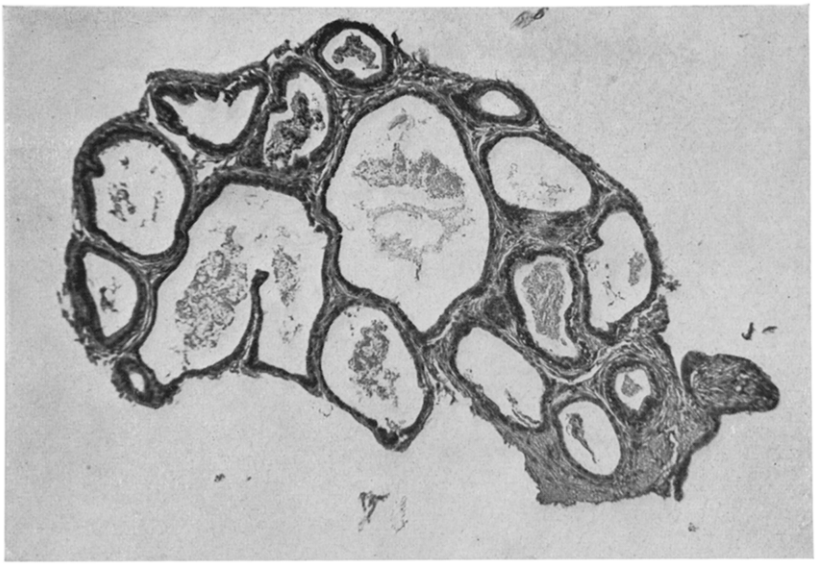

*Plate* 1. *Photomicrograph of fixed and stained organ culture of mouse ventral prostate* [45].

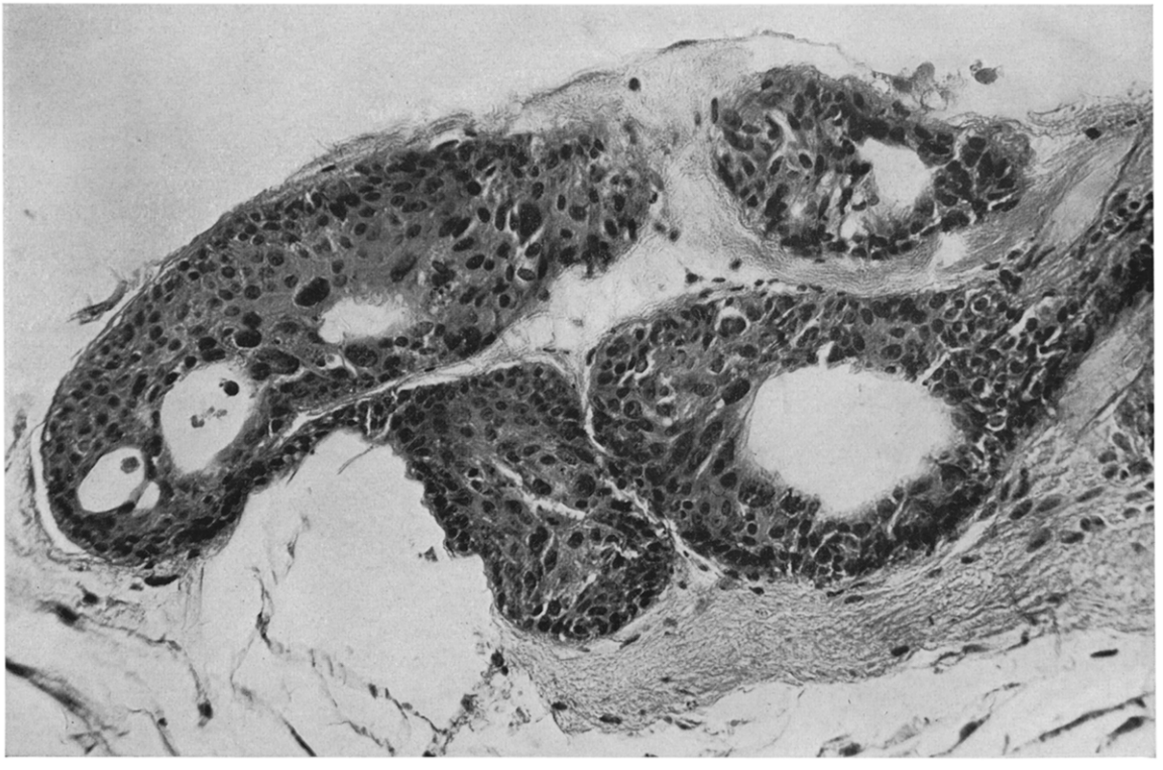

*Plate* 2. *Photomicrograph of fixed and stained organ culture of mouse ventral prostate treated with* 10 μg/*ml of* 1,2,5,6-*dibenzanthracene* [45].

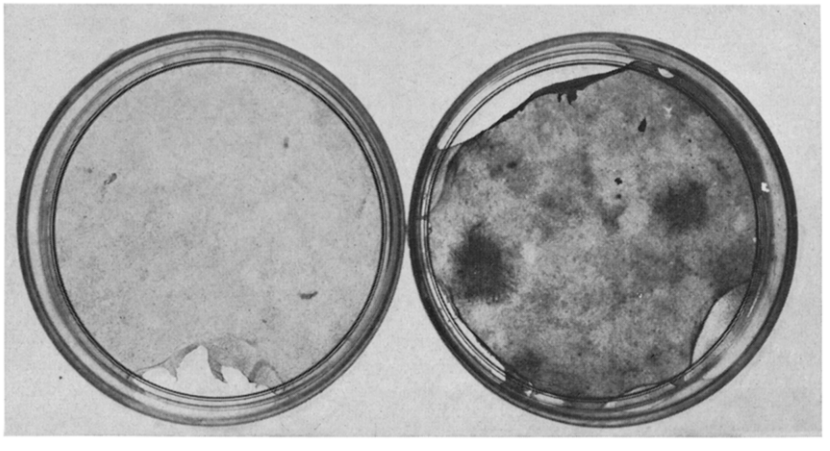

*Plate* 3. *Photograph of fixed and stained dishes of mouse prostate cells.*

(to face p. 166)

carcinogenesis *in vitro* has been achieved entirely in cell culture, and under conditions where spontaneous transformation does not occur [50].

It should be mentioned that while our studies were in progress other workers have reported varying degrees of success in obtaining chemical carcinogenesis *in vitro*. The first of these was by Berwald and Sachs [51] who obtained piled-up colonies of mouse and hamster embryo cells after treatment with benzpyrene. In mass cultures, tumors were produced by the untreated and treated mouse embryo cells, indicating a high rate of spontaneous transformation, and by the treated, but not by the untreated hamster embryo cells. These workers were also able to quantitate their experiments. Other non-quantitative experiments with hydrocarbons in various cell culture systems have been reported by Borenfreund *et al.* [52] and DiPaolo and Donovan [53], with nitroquinoline-N-oxide and its derivatives by Kuroki and Sato [54] and with N-nitrosomethylurea by Sanders and Burford [55]. Other papers from Sachs' laboratory will be discussed later.

Our system just described finally provided the basis for quantitation. When the control prostate cells were plated at a low density with or without a feeder layer and treated with 0·5% DMSO, they remained as a monolayer and gave the appearance of the left-hand dish of Plate 3 when fixed and stained after 2 weeks. By contrast, when the cells were treated for 6 days with 1 μg/ml of MCA and the cultures continued for 2 weeks more, densely-stained piled-up colonies were observed (Plate 3) and could be counted. Replicate dishes were scored for overall plating efficiency at 8 days to give a measure of the toxicity exerted by the hydrocarbon [56].

It then became necessary to determine whether individual piled-up colonies, if not fixed and stained, were capable of giving tumors. The results shown in Table 3 show clearly that each piled-up colony in a dish gave rise to tumors when the cells were injected into the brains of mice, whereas the monolayer areas of MCA-treated dishes did not [56]. This experiment fully justifies the scoring method that we use, and similar experiments have not been reported by others.

It was now time to determine whether the number of transformed colonies produced by hydrocarbons *in vitro* was related to their carcinogenic activities in mice. Using the quantitative system described above, the non-carcinogenic hydrocarbons 1,2,3,4-DBA, pyrene, and 3-fluoro-10-methyl-1,2-benzanthracene did not produce any piled-up colonies at any dose. On the other hand, as shown in Figs. 10–12, five carcinogenic hydrocarbons produced piled-up colonies in proportion to their carcinogenic activities [56]. It is also shown in Figs. 10–12 that there is no direct relation between the frequency of transforma-

*Table 3. Inoculation of individual piled-up colonies and monolayer areas into the brains of C3H mice**

| Dish No. | Type of area isolated | No. cells inoculated mouse | No. mice | Duration of observation days | Times of tumor appearance days | No. tumors |
|---|---|---|---|---|---|---|
| 1 | Monolayer | 1000 | 4 | 150 | — | 0 |
| 1 | Piled-up | 1000 | 3 | 90 | 30–90 | 3 |
| 1 | Piled-up | 100 | 3 | 150 | 30–150 | 2 |
| 1 | Piled-up | 10 | 3 | 150 | 36 | 1 |
| 2 | Monolayer | 1000 | 3 | 60 | — | 0 |
| 2 | Piled-up Colony 1 | 500 | 3 | 60 | 30–60 | 3 |
| 2 | Piled-up Colony 2 | 500 | 3 | 60 | 30 | 1 |
| 2 | Piled-up Colony 3 | 500 | 3 | 60 | 30 | 1 |
| 2 | Piled-up Colony 4 | 500 | 3 | 60 | 30 | 1 |
| 2 | Piled-up Colony 5 | 500 | 3 | 60 | 30–60 | 2 |
| 2 | Piled-up Colony 6 | 500 | 3 | 60 | 30–60 | 2 |

MCA at 1 μg/ml was added to each dish. Each experiment represents one 35 mm dish. For description, see text.
*[56].

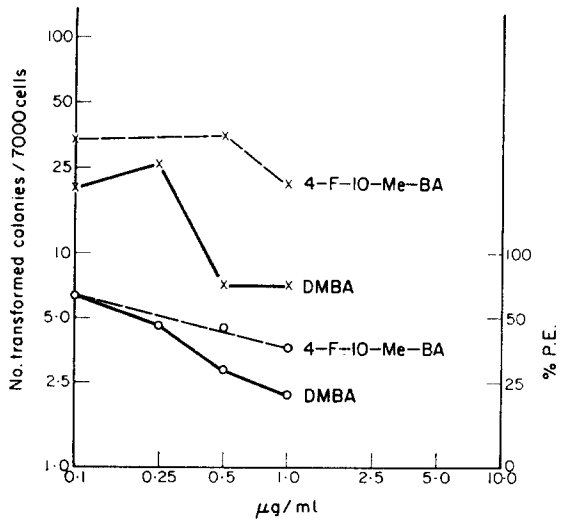


*Fig.* 10. *The transformation frequency and toxicity produced by* 4-*fluoro*-10-*methyl*-1,2-*benzanthracene and* 9,10-*dimethyl*-1,2-*benzanthracene* [56].

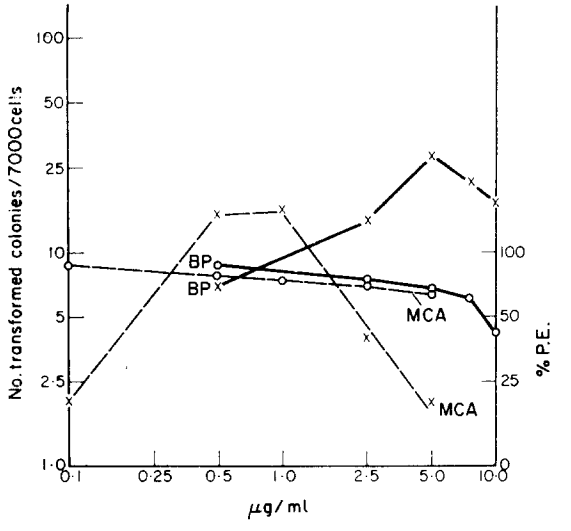


*Fig.* 11. *The transformation frequency and toxicity produced by* 3,4-*benzpyrene, and* 3-*methylcholanthrene* [56].

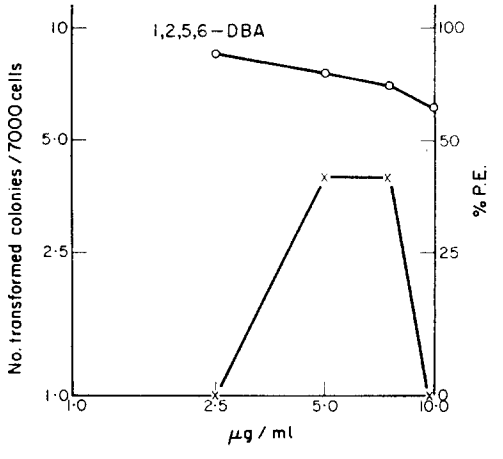


*Fig.* 12. *The transformation frequency and toxicity produced by* 1,2,5,6-*dibenzanthracene* [56].

tion and the toxicity produced by the hydrocarbons, in agreement with the findings of Huberman and Sachs [57]. The reason for the sharp decrease in transformation frequency with higher doses of carcinogens remains to be explained.

Experiments have also been carried out by treatment of the cells for only one day with methylcholanthrene, as shown in Table 4.

*Table* 4. *Transformation frequencies produced by one-day treatment with MCA* (10 μg/ml) *added at different times after plating**
(*B*1 *prostate cells were used at the 19th subculture*)

| Time of addition of hydrocarbon, days after plating | No. transformed colonies No. dishes | |
|---|---|---|
| | DMSO | MCA |
| 1st | 0/10 | 40/10 |
| 2nd | 0/10 | 27/10 |
| 3rd | 0/10 | 8/8 |
| 6th | 0/10 | 3/10 |
| 8th | 0/10 | 0/10 |

The scoring was done two weeks after the day of treatment with MCA.
*[56].

When the one day of treatment was shortly after plating, transformation did occur, but if the treatment with MCA was done on day 8 after a monolayer was formed, no transformation occurred. This observation is similar to that of Borek and Sachs [58] who, using X-rays for transformation, found a requirement for cell division to "fix" the transformed state.

We have recently demonstrated biochemically and clinically the remarkable sensitivity of cancer cells to the lethal effects of heat [59]. We have found (Fig. 13) that our transformed cells are much more easily killed by heat than our control cells, proving that the acquisition of heat sensitivity is an early consequence of carcinogenesis in this system [56]. How general this is, remains to be seen.

A rather puzzling aspect of this work is the question of the origin of our cells. The organ cultures treated with hydrocarbons show a degeneration of the connective tissue and a proliferation of the epithelial cells. Furthermore, the cell cultures derived therefrom exhibited an epithelial-like morphology. Yet the tumors, although they included a few carcinomas, were mostly sarcomas [48]. The cell lines used for the present quantitative studies usually also look epithelial in culture, but invariably give sarcomas [50, 56]. In an

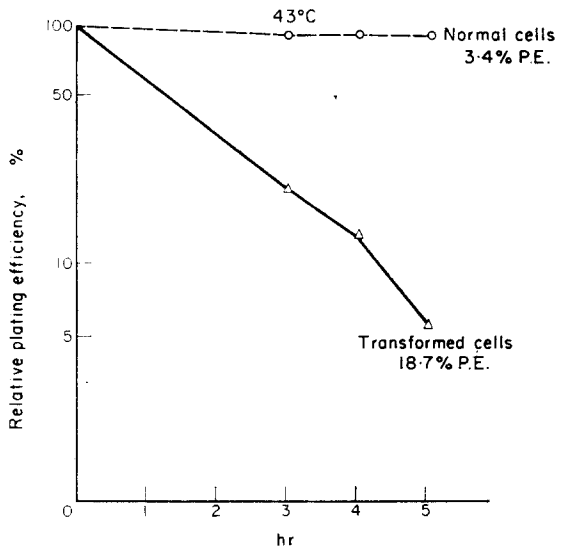


*Fig.* 13. *The lethal effect of treatment at* 43°C *of control and transformed prostate cells* [56].

attempt to ascertain the cell of origin, we had hoped to use acid-phosphatase as a specific enzymatic marker for prostatic epithelial cells, which it is in man. However, we found that the murine enzyme is not suitable as a marker [60]. Present studies on collagen synthesis (Fridland, Iype and Heidelberger, in preparation) suggest that these cells really are fibroblastic.

Thus far, the word "transformation" has been used in a purely operational sense with no mechanistic implications. Now the system was considered ready to be used to answer which of the 3 cellular mechanisms discussed above: direct transformation, selection of pre-existing malignant cells, or activation of an oncogenic virus obtain in this system. Very recent studies made possible by the consummate skill of Dr. Sukdeb Mondal using single cells have shown unequivocally that the process we study is a direct transformation and not a selection—the first time this has been proved in any system of chemical carcinogenesis [61]. Whether the direct transformation results from the direct interaction with some critical target in the cell or from the activation of an oncogenic virus is currently under study, but unfortunately the results are not yet at hand. Obviously, this is a critical matter, because one would design very different sorts of biochemical experiments depending on whether or not an oncogenic virus is involved in this mechanism of transformation.

We plan to utilize our *in vitro* system for appropriate biochemical and metabolic studies with respect to the binding of hydrocarbons to DNA, RNA and protein, to ascertain the biological role of the protein to which the hydrocarbons are bound, and in other ways to dissect the process of chemical carcinogenesis in a way that is not possible *in vivo*. Hopefully, we can come up with information that will be pertinent to the cellular and molecular mechanisms of hydrocarbon carcinogenesis. However, we must continually ask ourselves whether this system really is a good model for *in vivo* carcinogenesis. For example, our control cells are aneuploid (an invariable consequence of working with mouse cells) and cannot be termed normal. Is this aneuploidy a prerequisite for the malignant transformation that we observe? Can host immunological effects play a role in carcinogenesis, which could not be imitated in this system? As we continue with the system we must continually try to answer some of these questions.

You have kindly joined me in the peregrinations of a single investigator along the road to an understanding of hydrocarbon carcinogenesis. You will probably agree with me that we have not come a very long way since the time of Percival Pott. Yet new systems and techniques provide the tools for some undeniable progress in our understanding of this subject — an understanding that no longer needs to defer to the virologists, who also have a long way to go before the secrets of viral oncogenesis are fully understood. And who knows? Perhaps the chemical and the viral carcinogenesis people may be really working on the same subject.

In conclusion let me pose some questions that appear to me to be crucial for our understanding of this subject. Is an oncogenic virus involved in the action of carcinogenic hydrocarbons? If so, how is it activated? If not, what is the critical cellular target of the carcinogen? What is the chemical process of metabolic activation of hydrocarbons that leads to their interaction with cellular and molecular targets? If the mechanism does not involve the activation of a latent oncogenic virus, is it mutational or non-mutational? When these questions can be answered, prevention or prophylaxis should follow, and the road travelled by Pott, Cook, Kennaway, Haddow, Berenblum, Boyland, Brookes, Lawley and Lasnitzki shall finally reach its destination.

## SUMMARY

*The author has reviewed his research carried out over the past* 20 *years concerned with the cellular mechanisms of hydrocarbon carcinogenesis. The interaction of labeled carcinogenic hydrocarbons with the DNA, RNA, and proteins of mouse skin has been studied at various times after topical application, and the results correlated with the carcinogenic process. A quantitative system, employing cells derived from C3H mouse ventral prostate, has been developed for obtaining malignant transformation* in vitro *with carcinogenic hydrocarbons. A mechanism involving the selection of preexisting malignant cells by the carcinogen has been ruled out with this system.*

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# Effect of Local X-Irradiation on the Dissemination of Ehrlich Carcinoma Transplanted Intracerebrally

G. FRANCHI * and D. W. VAN BEKKUM
*Radiobiological Institute TNO, Lange Kleiweg 151, Rijswijk (ZH) The Netherlands*

PREVIOUS studies [1] have demonstrated that Ehrlich carcinoma transplanted into the brain of Swiss female mice disseminates into the lungs at a progressively increasing rate with the time. The presence of cancer cells in blood and lung of tumor-bearing animals was evaluated in a quantitative way by a biological assay [2].

To investigate whether the dissemination was due to a release of cancer cells from the primary tumor transplanted in the brain only or in addition to metastatic cells proliferating in the lung and recirculating in the blood, the following experiments were performed. Inbred Swiss/Rij-female mice were inoculated intracerebrally with Ehrlich carcinoma ($10^5$ cells in 0·01 ml) maintained in the ascites form according to a technique previously described [3]. Eight days after tumor transplantation the animals were divided at random in three groups of 16 mice each: the first group was used as a control, the second and the third group were treated under a light pentobarbital anaesthesia (35 mg/kg i.p.) with X rays (500 rads or 1000 rads) directed at the head of the animal while the rest of the body was protected by a lead shield.

A 250 kVp, 30 mA, Maxitron was used with the following radiation characteristics: half-value layer 2·1 mm Cu, dose rate 50 rads/min. The biological assay of cancer cells present in blood, lung, and brain was performed 1 hr and 4, 8 and 11 days after irradiation of the head. Blood clot or fragments or organs ($40\pm10$ mg) from mice bearing the intracerebral tumor were transplanted subcutaneously on both retroscapular sides of at least four normal mice. The growth of a tumor in the recipient animal within 30 days after the transplantation was considered proof that cancer cells were present in the blood or in the organs of the donor animals [1].

An attempt to establish a quantitative basis for the study of cancer dissemination was made by transplanting subcutaneously 10; 100; 1000; 10,000 cancer cells added to 40 mg blood, lung, brain, just before transplantation. The number of takes of the tumors were determined 30 days after the subcutaneous transplantation. A linear relationship exists between the percentage of takes expressed in probit and the logarithms of numbers of implanted cancer cells. On this basis a quantitative estimate of the number of cancer cells involved in the various phases of cancer dissemination is possible [2].

The results presented in Table 1 show that the percentage of tumor takes obtained by the subcutaneous transplantations of brain, blood and lung in normal mice decreases already 1 hr after X-irradiation.

At subsequent times the growth of the tumor transplanted into the brain and the dissemination in the blood and lung return toward the values present before the irradiation. At 19 days after the intracerebral-tumor transplantation only irradiated animals

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*Present address: Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62, 20157 MILANO, Italy.

*Table 1. Effect of X-Irradiation on growth and dissemination of Ehrlich carcinoma transplanted intracerebrally*

| Dose of X-rays | Time after irradiation | Brain | | Blood | | Lung | |
|---|---|---|---|---|---|---|---|
| | | % takes | cancer cells/g | % takes | cancer cells/ml | % takes | cancer cells/g |
| None | 1 hr | 100 | >2,500,000 | 75 | 100,000 | 75 | 400,000 |
| | 4 days | 100 | >2,500,000 | 75 | 100,000 | 87 | >400,000 |
| | 8 days | 100 | >2,500,000 | 87 | 500,000 | 87 | >400,000 |
| | 11 days* | | | | | | |
| 500 rad | 1 hr | 37 | 60,000 | 37 | 4000 | 12 | 300 |
| | 4 days | 62 | 350,000 | 12 | 250 | 25 | 2,700 |
| | 8 days | 100 | >2,500,000 | 60 | 11,000 | 75 | 400,000 |
| | 11 days | 100 | >2,500,000 | 62 | 30,000 | 75 | 400,000 |
| 1000 rad | 1 hr | 0 | <10,000 | 12 | 250 | 12 | 300 |
| | 4 days | 37 | 60,000 | 12 | 250 | 25 | 2,700 |
| | 8 days | 100 | >2,500,000 | 37 | 4000 | 50 | 325,000 |
| | 11 days | 100 | >2,500,000 | 75 | 100,000 | 75 | 400,000 |

*All the animals died with large brain tumors.

survived while the controls died as a result of the extent of tumor growth at the primary site. These findings suggest that the dissemination of cancer cells in blood and lung mainly depends on the availability of cancer cells in the brain. Furthermore it is evident that when the cancer growth in the brain is blocked by the irradiation and presumably the release of cancer cells is inhibited, the lung is capable of destroying a large number of malignant cells in a relatively short time. This supports the view that in our model cancer cells present in the lung do not contribute to the circulating cancer cells, but that the latter are mostly derived from the primary tumor in the brain. Since the Ehrlich carcinoma transplanted in Swiss mice is allogenic, it may be possible that the continuous destruction of disseminating cancer cells is due to an immunological reaction.

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## SUMMARY

*The dissemination of cancer cells in blood and lungs from Ehrlich carcinoma transplanted into the brain of mice has been investigated at different times after treatment with X-rays* (500 *rads or* 1000 *rads*) *directed at the head of the animals. One hr after irradiation the malignant cells in blood and lungs decreased proportionally to the inhibition of the "primary tumor". These findings suggest that the cancer cells present in lung do not contribute in this experimental model to the circulating cancer cells. It is evident that cancer cells present in lung are rapidly destroyed when the supply is blocked.*

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# A Study of Some Tumours of Human Origin—I

## Chromosomes of Rat Tumour (HR 18) and Mouse Tumour (HM 18) Obtained by Hetero-Transplantation of a Human Melanocarcinoma

N. C. POPESCU, LIDIA CIOLOCA, FLORENTINA LICIU and I. ENCUT

*Laboratory of Cellular Biology, Oncological Institute, Bucharest, Romania*

### INTRODUCTION

A HUMAN melanocarcinoma was heterotransplanted in rat and mouse by intra-embryonic method and in hamster by inducing tolerance [1]. The intra-embryonic inoculation method was successfully used in previous experiments [2–4]. The tumours developed on the xenogeneic hosts presented an aggressive behaviour, a capacity of invading growth and lung metastasis (especially in the rat host), similarly to other transplantable tumours of human origin [5, 6].

Our aim was to establish whether or not the tumours obtained by inoculating human tumoural material in xenogeneic hosts, preserve their human characteristics; for this purpose we studied the cytogenetic properties of the tumours obtained in rat and mouse.

### MATERIAL AND METHODS

Details on the methods and the materials used were given in a previous work [1].

Nevertheless, we will revert to it to point out that the tumour tissue was harvested from the patient D.I., aged 58, presenting a melano carcinoma of the shank, who, despite the treatments applied, developed inguinal, iliac and hepatic metastases as well as cancer ascites.

The material used for heterotransplantation was derived from the inguinal tumour which, upon microscopical examination, proved to be the massive lymph node metastasis of an abundantly pigmented melanocarcinoma.

The passages were carried out in embryos of WAG rats according to a technique already described [1] and in 12-days AKR mice that were previously injected with tumour cell suspension (reticulosarcoma) prepared from another case in our clinic.

The chromosome preparations were carried out when tumours reached convenient size according to a technique described by Nachtigal *et al.* [7] and Popescu *et al.* [8] including trypsinization (trypsin DIFCO 0·2%), hypoton treatment (sodium citrate 0·75%) and a number of 5 fixations in acetic acid ice-cold methanol 1/3. The slides were flame-dried and stained with Giemsa.

The tumours obtained bear the number 18 and the initials representing the donor and the tumoural recipient are:

$H$ = human, $R$ = rat, $M$ = mouse.

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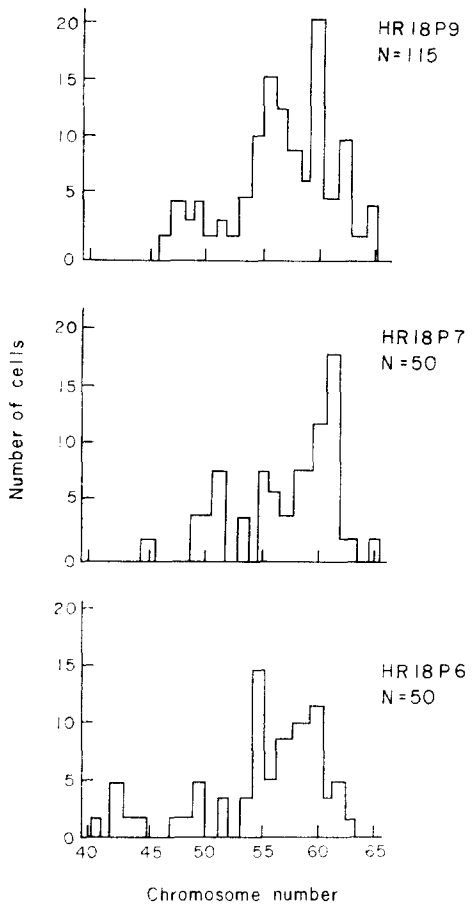


*Fig. 1. Chromosome distribution of tumours obtained in rat (HR 18, passages 6, 7 and 9). N=number of metaphases analyzed.*

## RESULTS

The normal karyotype of WAG rat includes 42 chromosomes ranged according to the suggestion of Hungerford and Nowell [9] and that of AKR mouse includes 40 telocentric chromosomes [10] Figs. 3 and 6.

For rat-tumour cells the adherence to the ranging scheme of normal karyotype was not possible; the size and form of chromosomes were used as criteria; 4 groups were established: subtelocentrics (A), submetacentrics (D), telocentrics (B) and metacentrics (C) (Fig. 5). The same was used for other rat tumours too [11, 12].

Three rat tumours were analyzed at passages 6, 7 and 9 (HR 18) and a mouse tumour (HM 18) upon its appearance.

We would point out that no human metaphase could be identified among all metaphases analyzed, the constitution of the tumours obtained in rat and mouse presenting the characteristics of the host animal.

The analysis of the karyotype and histogram (Figs. 1, 4 and 5) revealed a stem-line ranging between 54–62 chromosomes, a remarkable karyotypic uniformity—as showed by a similar distribution in groups (Table 1)—and the presence of the following markers: M1A = (in the group of subtelocentric chromosomes with secondary constrictions on the long arms), M1D (in the group of submetacentric chromosomes), M1B (in the group of telocentric chromosomes) (Figs. 4 and 5).

The HM 18 mouse tumour presented the cytogenetic characteristics of the host too. The mouse tumour appeared on the grafting site of the human tumour and became evident on the fifth day after grafting when the mice were 17 days old.

The analysis of over 700 metaphases and of 100 absolutely counted metaphases revealed the following characteristics: complete absence of human metaphases, a chromosome distribution including apparently normal diploid cells with 40 telocentric chromosomes, hypodiploid and tetraploid cells (Fig. 2). Most of the tetraploid cells presented biarmed chromosomes, unusual in the species, while the near diploid cells, with a single exception, did not present such chromosomes. Eighty-four per cent of the analyzed cells presented an average of 2 or 3 biarmed metacentric chromosomes (Table 2) larger or of the same size as the first telocentric one, and a varying number of very small accrocentric chromosomes, smaller than the smallest normal telocentric one (Fig. 7). In this tumour we also noticed the presence of a large accrocentric marker chromosome bearing secondary constrictions (M1) (Fig. 7).

## DISCUSSION

The chromosomal species specific differences between man and rat and mouse as xenogeneic host, enabled us to notice that by inoculating the same surgical human tumoural material, tumours with a chromosome constitution specific to the host, developed.

"Inducing" of host tumours in the same species was also obtained by Klausner and Richards through heterotransplantation of a HT37 human carcinoma [13] and by Costăchel *et al.*, through the heterotransplantation of a spontaneous hamster melanoma [2].

Also Popp *et al.*, using intra-embryonic inoculation, heterotransplanted the spontaneous H 10 hamster tumour in rat and obtained tumours with 96% host cells [4].

The aggressive behaviour of the tumours on xenogeneic host and the easiness in the heterotransplantation of this type of human tumours [14] were the decisive factors in the investigation of their nature and cytogenetic constitution.

The same goal was also pursued by Haemmerli *et al.* in the analysis of a GW 127 tumour

*Table 1. Chromosome distribution by groups of heteroploid cells karyotypically analyzed in tumours (HR 18) obtained in rats*

| Cell No. | Chromosome type | | | | Marker chromosomes | | | No. of chr. | Tumour No. |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | M1A | M1B | M1D | | |
| 1 | 9 | 14 | 11 | 7 | — | — | — | 44 | 1 |
| 2 | 11 | 17 | 10 | 8 | — | 1 | 1 | 46 | 1 |
| 3 | 10 | 17 | 10 | 11 | — | 1 | 1 | 48 | 3 |
| 4 | 8 | 19 | 10 | 11 | — | 1 | 1 | 48 | 2 |
| 5 | 15 | 16 | 8 | 10 | — | 1 | 1 | 49 | 1 |
| 6 | 12 | 13 | 15 | 11 | 1 | — | 1 | 51 | 2 |
| 7 | 10 | 20 | 14 | 8 | — | 1 | 1 | 52 | 3 |
| 8 | 10 | 20 | 13 | 11 | 1 | 1 | 1 | 54 | 3 |
| 9 | 11 | 22 | 12 | 12 | 1 | 1 | 1 | 57 | 2 |
| 10 | 13 | 20 | 12 | 12 | 1 | 1 | 1 | 57 | 2 |
| 11 | 14 | 18 | 11 | 14 | — | 1 | 1 | 57 | 3 |
| 12 | 14 | 17 | 12 | 14 | — | 1 | 1 | 57 | 3 |
| 13 | 11 | 21 | 13 | 12 | 1 | 1 | 1 | 57 | 3 |
| 14 | 12 | 18 | 14 | 14 | 1 | — | 1 | 58 | 2 |
| 15 | 13 | 19 | 13 | 12 | 1 | — | 1 | 58 | 2 |
| 16 | 10 | 20 | 15 | 13 | 1 | — | 1 | 58 | 3 |
| 17 | 10 | 22 | 14 | 12 | 1 | 1 | 1 | 58 | 3 |
| 18 | 11 | 17 | 14 | 14 | 1 | 1 | 1 | 58 | 3 |
| 19 | 14 | 22 | 12 | 10 | 1 | 1 | 1 | 58 | 3 |
| 20 | 15 | 19 | 15 | 10 | — | 1 | 1 | 59 | 1 |
| 21 | 11 | 21 | 17 | 11 | 1 | — | 1 | 60 | 1 |
| 22 | 14 | 21 | 15 | 11 | — | 1 | 1 | 61 | 1 |
| 23 | 14 | 23 | 12 | 12 | 1 | 1 | 1 | 61 | 1 |
| 24 | 16 | 18 | 12 | 16 | 1 | 1 | 1 | 62 | 3 |
| 25 | 15 | 17 | 12 | 16 | 1 | 1 | 1 | 62 | 2 |
| 26 | 14 | 18 | 14 | 16 | 1 | 1 | 1 | 62 | 3 |

HR 18 P6=Tumour No. 1; HR 18 P7=Tumour No. 2; HR 18 P9=Tumour No. 3.

*Table 2. Distribution of biarmed chromosomes and small telocentrics in 25 representative cells of tumour HM 18*

| Number of chromosomes | Biarmed chromosomes | Small telocentrics |
|---|---|---|
| 39 | 1 | — |
| 46 | 2 | — |
| 47 | 1 | 4 |
| 52 | — | 5 |
| 56 | 2 | 5 |
| 57 | 1 | 5 |
| 63 | 2 | 5 |
| 66 | — | 6 |
| 70 | 3 | 3 |
| 72 | 1 | 4 |
| 73 | 2 | 6 |
| 74 | 6 | 6 |
| 76 | 2 | 5 |
| 77 | 2 | 4 |
| 78 | 1 | 3 |
| 79 | 3 | 5 |
| 80 | 2 | 6 |
| 81 | 3 | 4 |
| 82 | 2 | 5 |
| 83 | 2 | 6 |
| 84 | 4 | 5 |
| 85 | 2 | 3 |
| 86 | 2 | — |
| 91 | 2 | 8 |
| 97 | 2 | 7 |

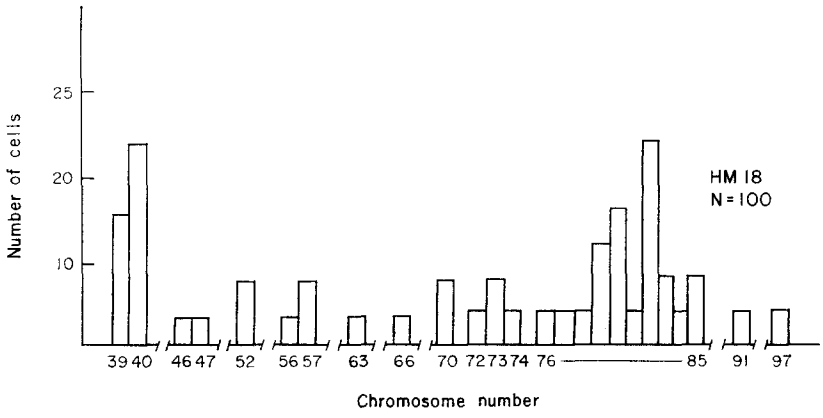


*Fig. 2. Chromosome distribution of tumours obtained in mouse (HM 18). N=number of metaphases analyzed.*

[5] obtained in hamster by Goldenberg, from an ovary carcinoma in the jugal pouch [6].

The GW 127 tumour, similarly aggressive on the xenogeneic host, proved to be chromosomally specific to the hamster [5]. Concerning the analysis of antigens Haemmerli demonstrated the absence of human antigens in the GW 127 tumour while Goldenberg and Götz [15], on the basis of their cytogenetic and serologic results, as well as on the comments of the results obtained by Haemmerli *et al.*, expressed their doubts as to the possibility of a complete absence of the human genome and antigens in the GW 127 tumour. They asserted the existence of both human and hamster antigens and that "no qualitative difference could be demonstrated with regard to the human antigen present in H.Ad. No. 1 and GW 127" (H.Ad. No. 1 is a tumour that retains its human chromosomal constitution on the heterologous host [16]).

The fact that the GW 127 tumour grows in hamster, a species which chromosomally is similar to man, rendered the cytogenetic analysis somewhat difficult, the study of late replication chromosomes being also necessary [5]. In our experiments the host species being chromosomally different from man, we were able to notice the absence of the human metaphases, thus having an indirect evidence of the absence of human genome and antigens.

Despite the fact that the heterotransplantation method was not similar to the one used for GW 127 tumour [5, 6], the way such tumours develop might be the same.

According to Haemmerli *et al.*, this type of tumours are either spontaneous or induced of the hamster, while Goldenberg *et al.* support the hypothesis of the interspecies hybridization between tumour cells and normal host cells [15].

Our findings did not give any evidence to support the hybridization hypothesis. The karyotype of HR 18 tumour contains new chromosomes unusual to the species, but a similar constitution was also found in other rat tumours we have analyzed [11, 12].

The HM 18 tumour studied upon its emergency and not after many passages also presented biarmed new chromosomes and small accrocentric ones, which were not found in the normal karyotype. It is difficult to account for the formation of the small telocentric chromosomes described by Stich and Wakoning in spontaneous leukaemia of AKR mice [17]. The biarmed chromosomes frequently appear under the action of different agents (18–21) and probably derive from one armed chromosome according to the mechanism described by Hsu [22] or Lithner and Pontén [23].

In most biarmed chromosomes the two arms were equal, originating from isochromosomes and not by random fusion between two telocentrics.

The interspecies hybridization conclusively demonstrated *in vitro* [24–26] might also take place *in vivo* but the resulting cells having a lower metabolic stability and a higher sensitivity to tumoural selection pressure are liable of an early disappearance.

Without eliminating any possibility of hybridization, we feel inclined to consider the tumours in our experiment, as well as others of the same type, as induced tumours of the host. There may exist in tumour implants certain stimulating agents which act as oncogenic agents in the host own tissues. There are some indications that these agents might be of viral origin. The present experiment may be related to that of Kirsten and Dominguez [27] who have heterotransplanted leukaemic cells of AKR mouse in new born rats. The chromo-

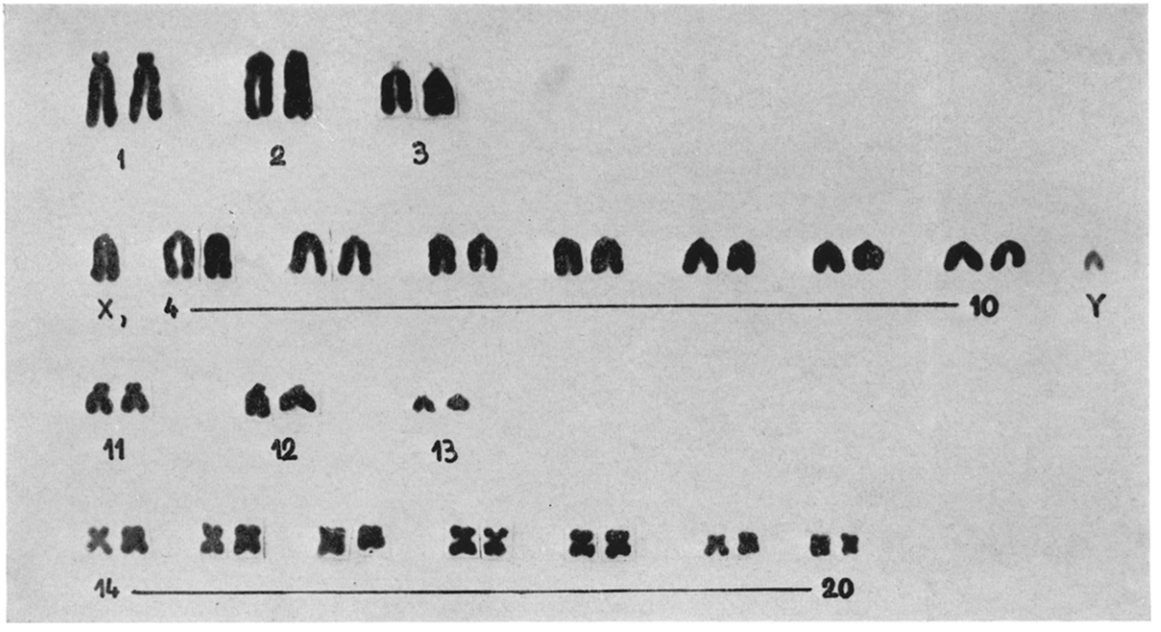


*Fig. 3. Normal karyotype of Wistar rat prepared from bone marrow of a male animal.*

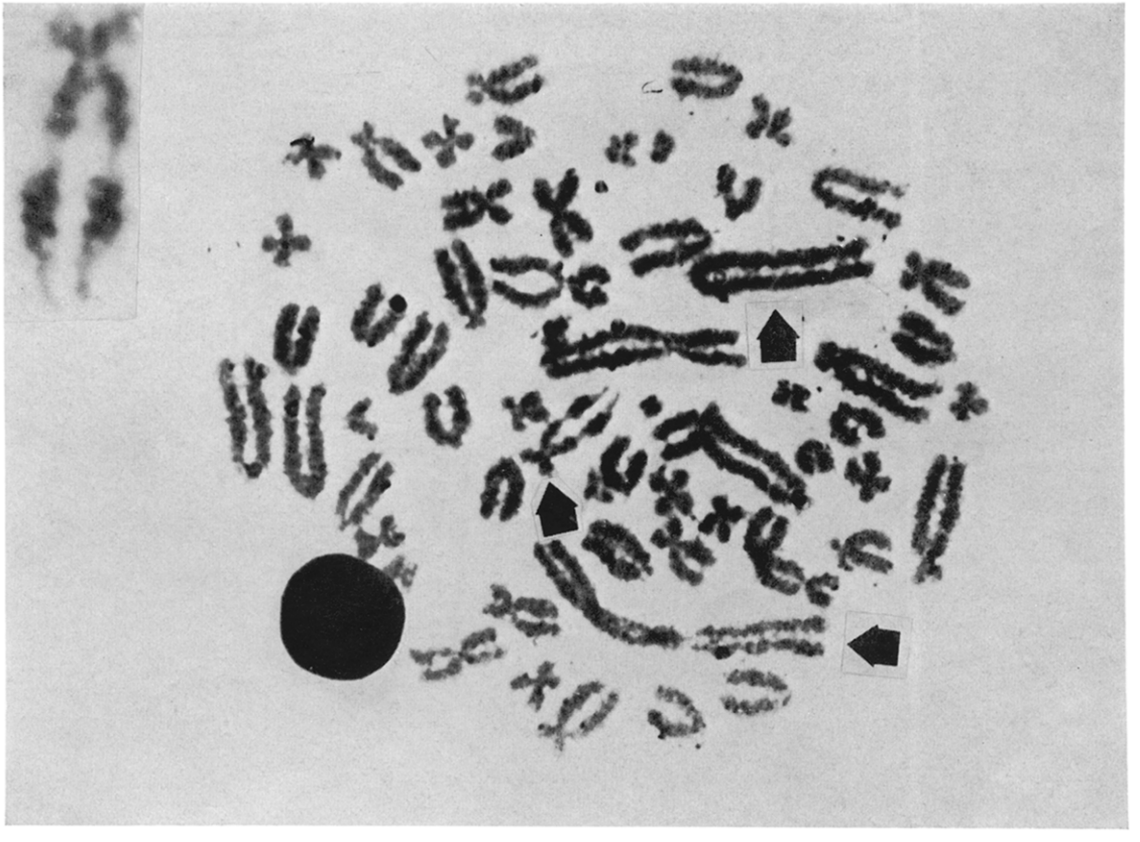

*Fig. 4. Metaphase with 61 chromosomes of tumour HR 18 P6. Note. The metaphase presents the characteristics of the host. Three markers indicated by arrow (M1A), (M1D), (M1B). Marker (M1S) bearing secondary constructions at a higher level of magnifying.*

(to face p. 178)

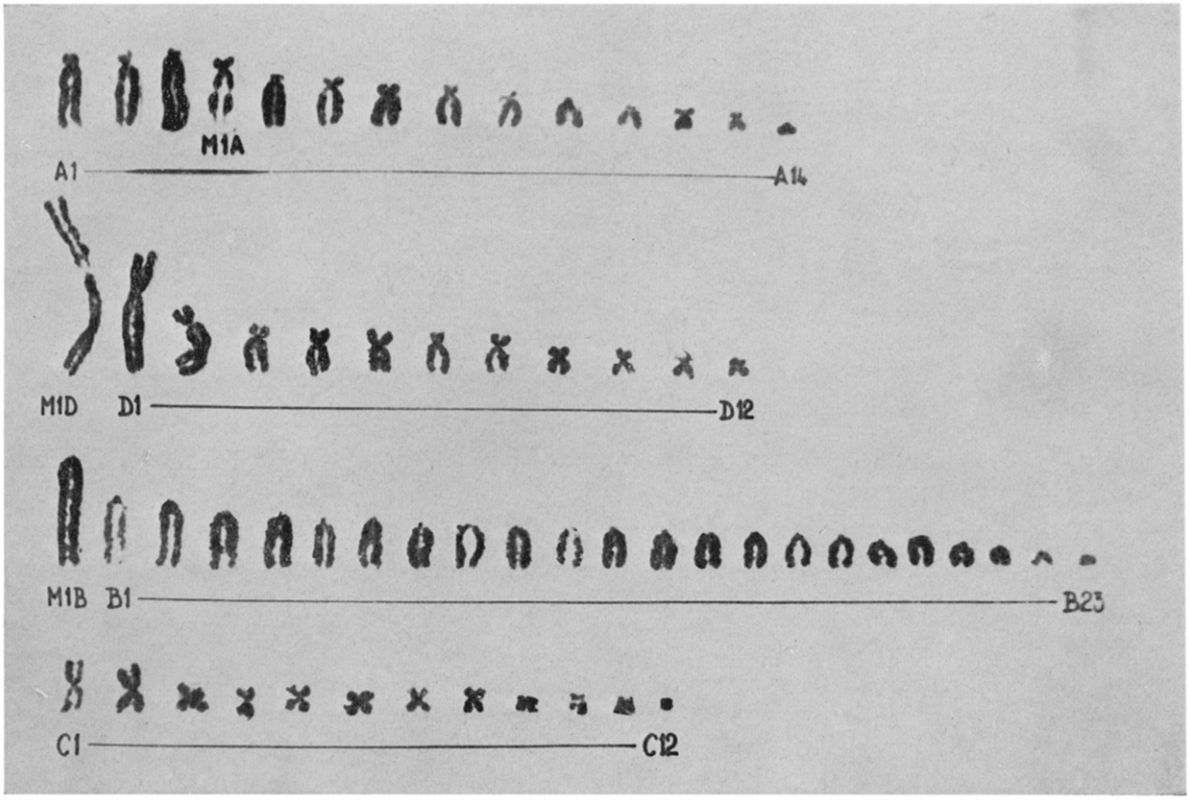


*Fig.* 5. *Karyotype of the metaphase of Fig.* 4, *ranged according to shape and size of the chromosomes.*

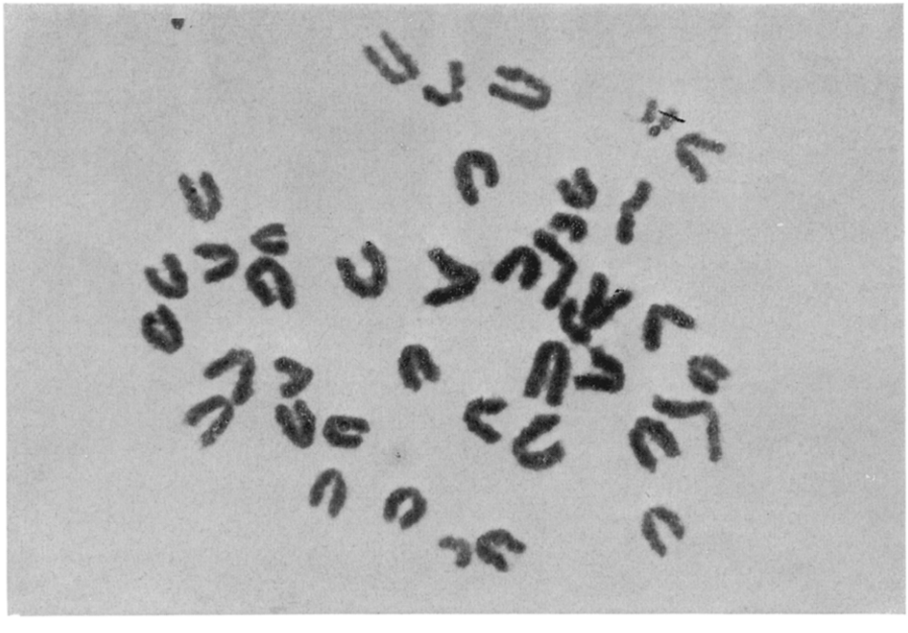

*Fig.* 6. *Apparent normal metaphase with* 40 *telocentric chromosomes obtained from the tumour developed in mouse* (*HM* 18).

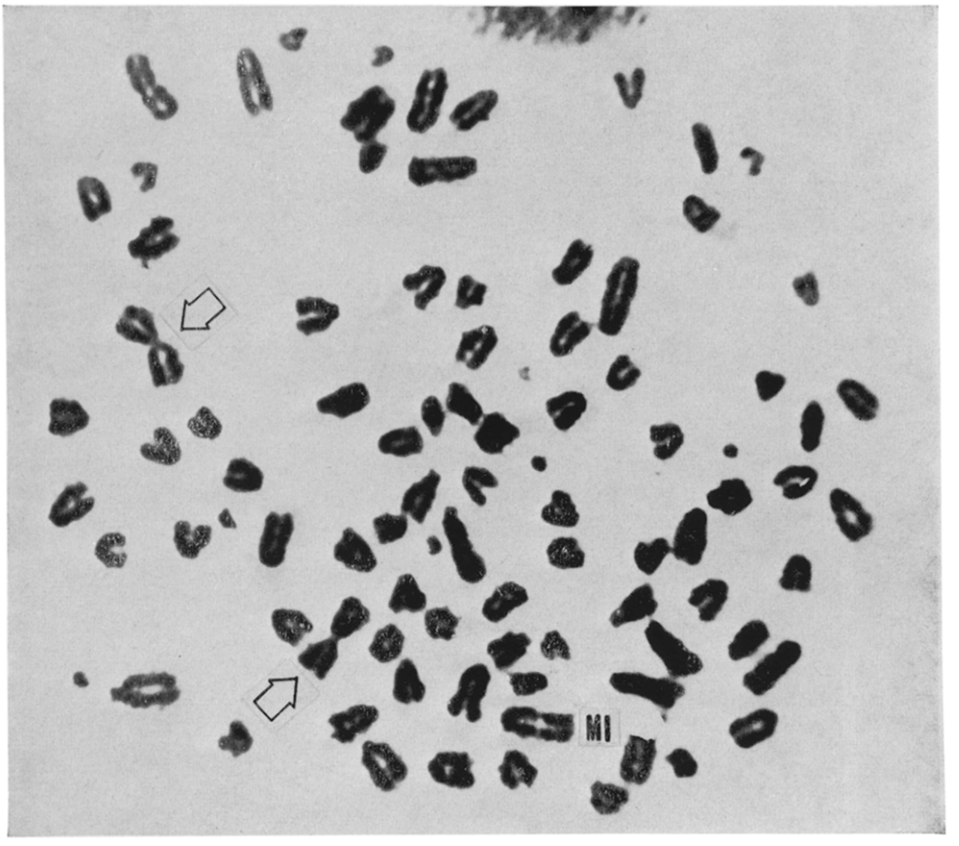


*Fig. 7. Metaphase with 91 chromosomes obtained from the tumour (HM 18). Note two biarmed chromosomes indicated by arrow, a marker chromosome (M1) bearing secondary constructions and 8 very small telocentric chromosomes.*

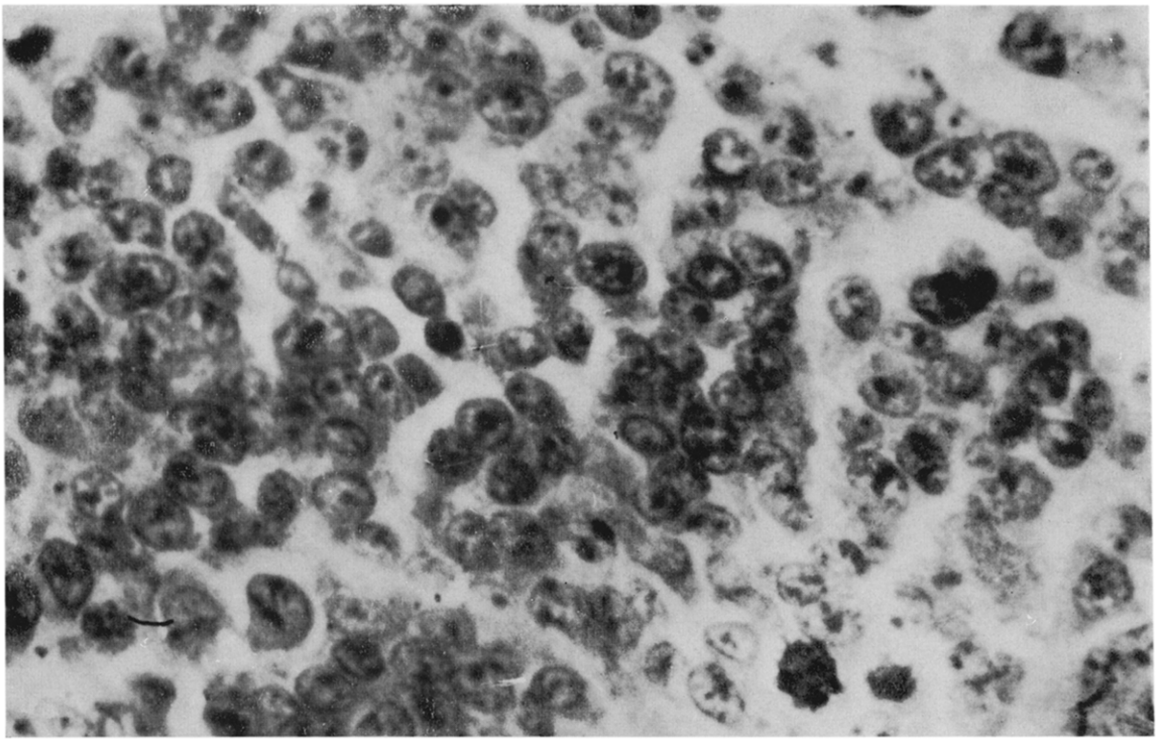

*Fig. 8. Histological aspect of HR 18 tumour at the 9th passage on rat.*

some analysis showed that the early form presented a mouse karyotype while in the advanced forms the cells had a rat karyotype. They obtained the same results when using the acellular filtrate. Recently, the heterotransplantation of a new human melanocarcinoma in rat and hamster was obtained in our Institute. The electron microscopic analysis revealed the presence of some viral particles in the hamster tumour [28] and, chromosomally, this was a typical hamster tumour [29]. Also these tumours were particularly aggressive on the xenogeneic host.

Hence we consider these tumours of great interest and further investigations might be useful in establishing their nature and tumoural evolution.

**Acknowledgements**—We wish to thank Mrs. Elena Marinescu, Mrs. Amelia Hera and Mrs. Elena Vlaic, for their technical assistance.

## SUMMARY

*The HR* 18*-rat tumour and the HM* 18 *mouse tumour obtained by the heterotransplantation of a human melanocarcinoma* [1] *evidenced an aggressive behaviour on the heterologous host.*

*The analysis of their chromosome constitution supplied information concerning the nature of these tumours.*

*Both rat and mouse tumours presented a chromosome constitution specific to the host animal.*

*The possibilities concerning the formation of these tumours are discussed.*

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# A Study of Some Tumours of Human Origin—II

## Chromosome and Late Replication Analysis of Parental Tumours, Metastasis and Ascites of the HR 18 Rat Tumour

N. C. POPESCU, LIDIA CIOLOCA and E. OLĂIANOS

*Laboratory of Cellular Biology, Laboratory of Nuclear Medicine, Oncological Institute, Bucharest, Romania*

### INTRODUCTION

HR 18 RAT TUMOURS obtained by the heterotransplantation of a human melanocarcinoma [1] were propagated in solid form, transformed in permanent ascites line [2] according to a technique already described [3], and heterotransplanted in AKR mice.

The HR 18 tumour, aggressive on rat, had similar manifestations on AKR mouse, also developing lung metastases.

Comparative chromosome analysis of parental tumours, lung metastasis and ascites of HR 18 rat tumour was performed.

DNA chromosomal late replication of ascites cells were studied by means of autoradiography.

### MATERIAL AND METHODS

The chromosome preparations for solid tumours and lung metastases were performed by usual technique [1]. For the chromosome preparation from ascites, the fluid was harvested from Wistar rats, on the 6th day after intraperitoneal inoculation.

The colchicine solution in concentration of 5 μg/g was i.p. administered and after 2 hr the animals were sacrificed. The harvested ascitic fluid was centrifuged for 10 min at 850 **g** and the sediment resuspended in hypotonic solution of Na citrate 0·75%. Two ascites were analyzed, 115 metaphases accurately counted.

The following tumours and lung metastases were studied:

T No. 1 HR 18 tumour in rat, passage 6, described in the previous paper [1].

T No. 2 Lung metastasis appearing in No. 1 tumour bearing rat.

T No. 3 Tumour obtained by intraembryonic heterograft of HR 18 P9 tumour in mice of the AKR strain.

T No. 4 Lung metastasis appearing in No. 3 tumour bearing animal.

T No. 5 Tumour obtained by retrograft of No. 3 tumour in WAG rat.

*Autoradiography*

For labelling, the ascitic cells were cultivated for 3 days in TC 199 medium (DIFCO) supplimented with calf serum and antibiotics. The tritiated thymidine $^3$HTdR (Amersham England) was administered in a concentration of 0·5 μC/ml culture medium for 5 hr, and the colchicine solution was added 2 hr prior to culture harvesting.

For the DNA replication study, 100 metaphases and 20 karyotypes prepared from metaphases before and after removal of the grains, were equally analyzed. The removal of

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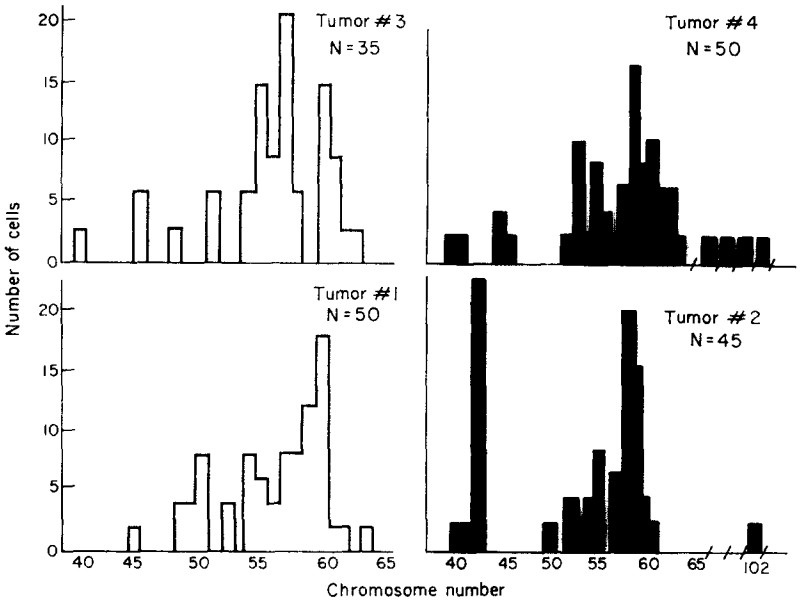


*Fig.* 1. *Chromosome distribution of parental tumours and lung metastases (HR* 18*) of rat and mouse*
*N=number of metaphases analyzed.*
*In black=distribution in lung metastases.*

the grains was carried out according to the method of Bianchi *et al.* [4], modified. The karyotype arrangement was performed according to the shape and size of the chromosomes.

## RESULTS

The tumour 1 [1] presents a cellular population having a hypotetraploid level, a remarkable chromosomal uniformity and a constitution specific to rat, including 3 characteristic markers [1].

Except for the high percentage of apparently normal diploid cells (Fig. 1) the lung metastasis of the same animal presented a similar chromosomal picture (Fig. 3). By intra-embryonic heterograft of a HR 18 P9 tumour in AKR mice, the chromosome constitution of the donor tumour was retained (Fig. 4). The heterografted animals also presented lung metastases within a short-time span.

Neither in this case chromosomal differences between parental tumours and lung metastases could be identified (Fig. 5).

A high number of tetraploid cells were present in the No. 4 tumour and No. 5 tumour obtained by retrografting of No. 3 tumour in rat (Fig. 1).

*Chromosome analysis and late-replication pattern of cultured HR* 18 *ascites cells*

The extemporaneous microscopical examination of ascites cells revealed the existence of some round cells (20 on HE and MGG stained smears) beside some giant cells both types

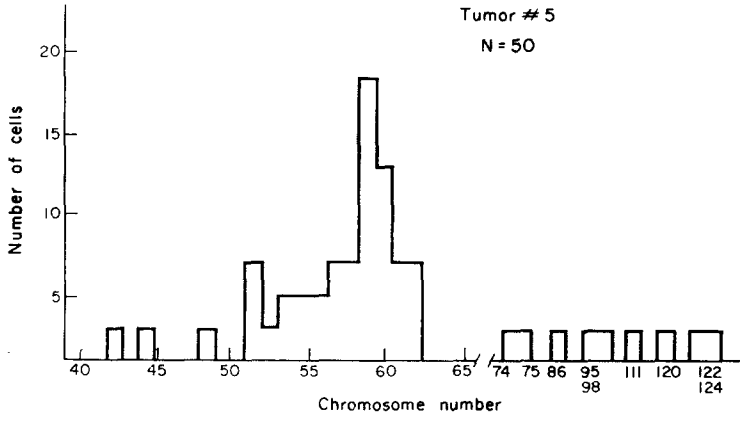


*Fig.* 2. *Chromosome distribution of the tumour obtained by retrograft in rat.*
*N=number of metaphases analyzed.*

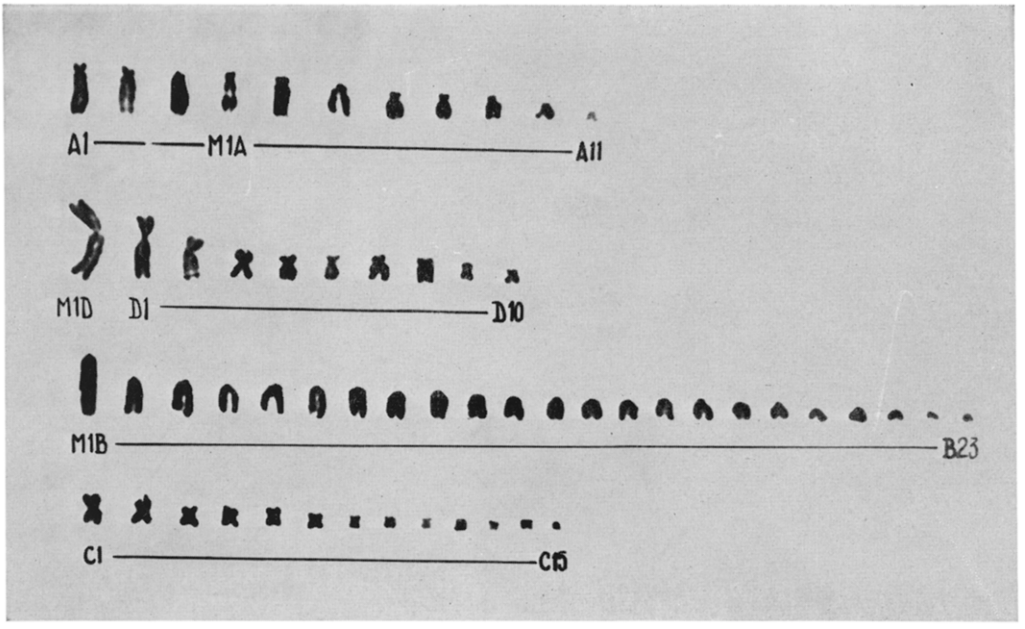


*Fig.* 3. *Karyotype of a metaphase with* 59 *chromosomes obtained from a lung metastasis in rat* (*No.* 2). *Note the presence of the markers characteristics to the parental tumour* (*M*1*A*) (*M*1*D*) *and* (*M*1*B*).

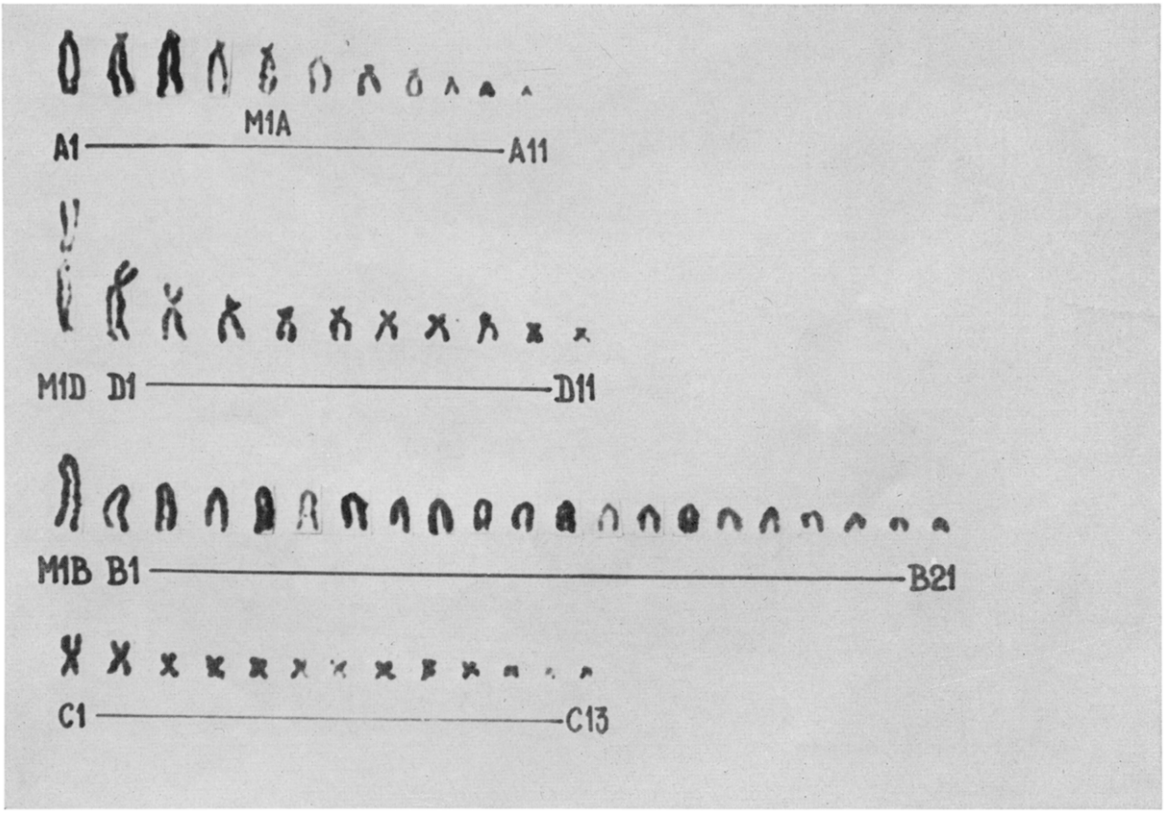


*Fig.* 4. *Karyotype with* 56 *chromosomes of the tumour* (*No.* 3) *obtained in AKR mouse by heterografting HR* 18 *tumour shows that it belongs to the donor.*

(to face p. 182)

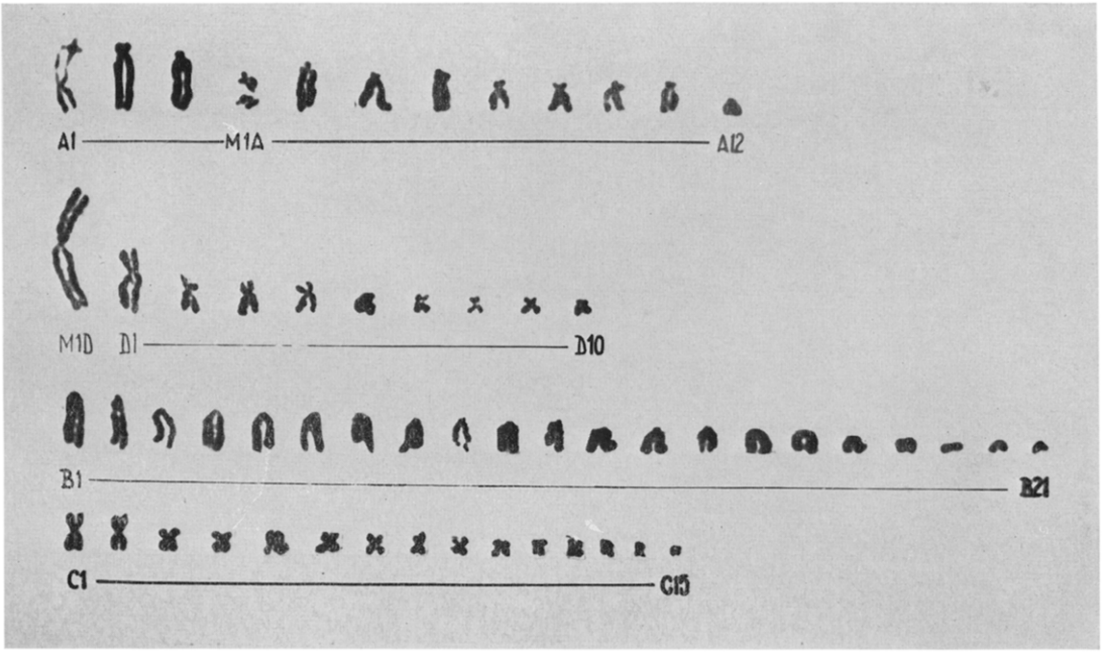


*Fig.* 5. *Karyotype of a metaphase with* 58 *chromosomes obtained from a lung metastasis* (*No.* 4) *appearing in mouse. A karyotypic picture similar to that of the parental tumour cells.*

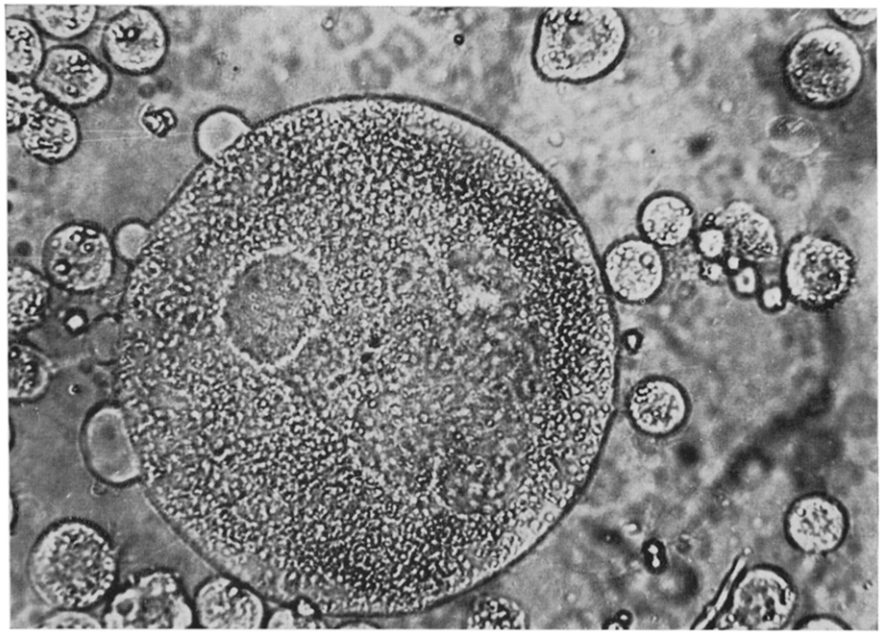

*Fig.* 6. *HR* 18 *ascitic cells on extemporaneous preparation by phase contrast. Small round cells and a giant cell with refringent grains and cytoplasmic excrescences.*

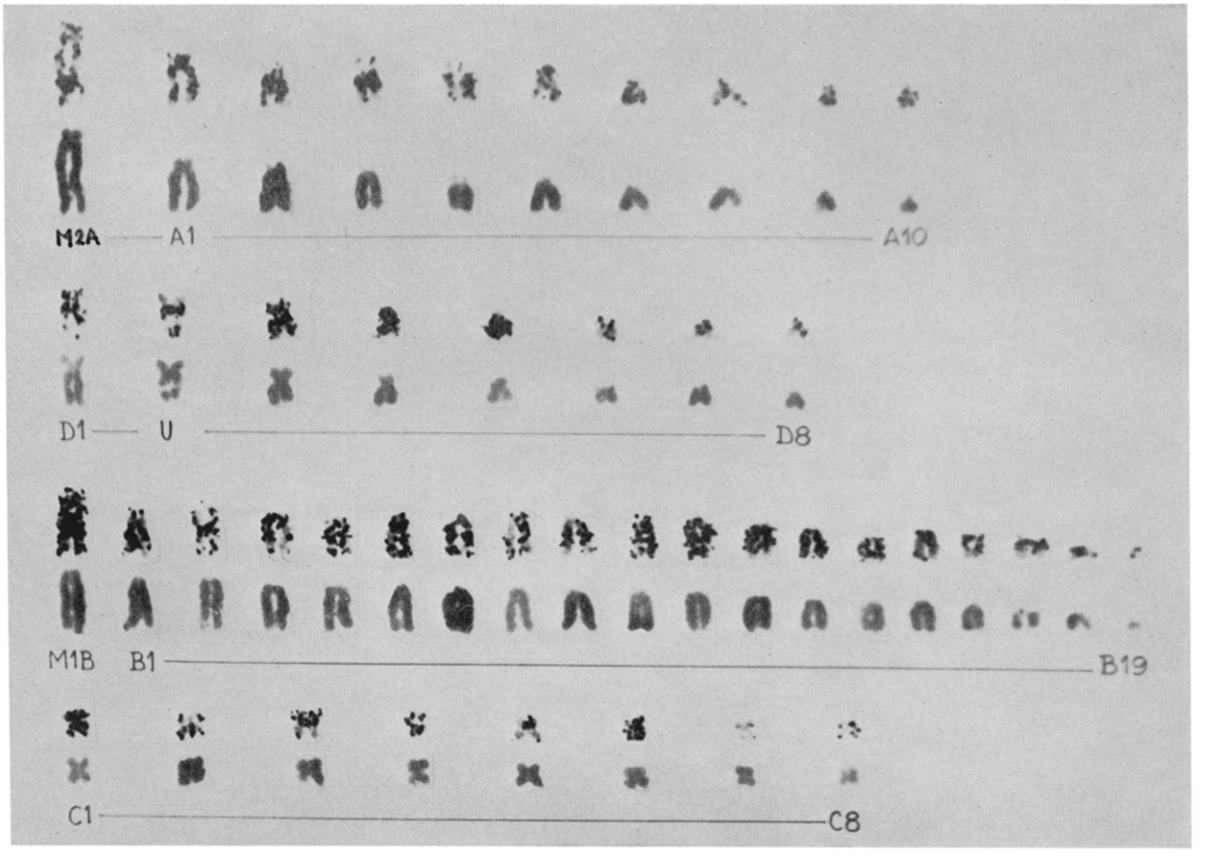


*Fig.* 8. *Karyotype of an ascitic cell with* 45 *chromosomes with most chromosomes labelled, marker (M2A) more strongly labelled at its extremities, a free labelling chromosome (U) in the region of secondary constrictions and a* 6*th metacentric chromosome (group C), unlabelled.*

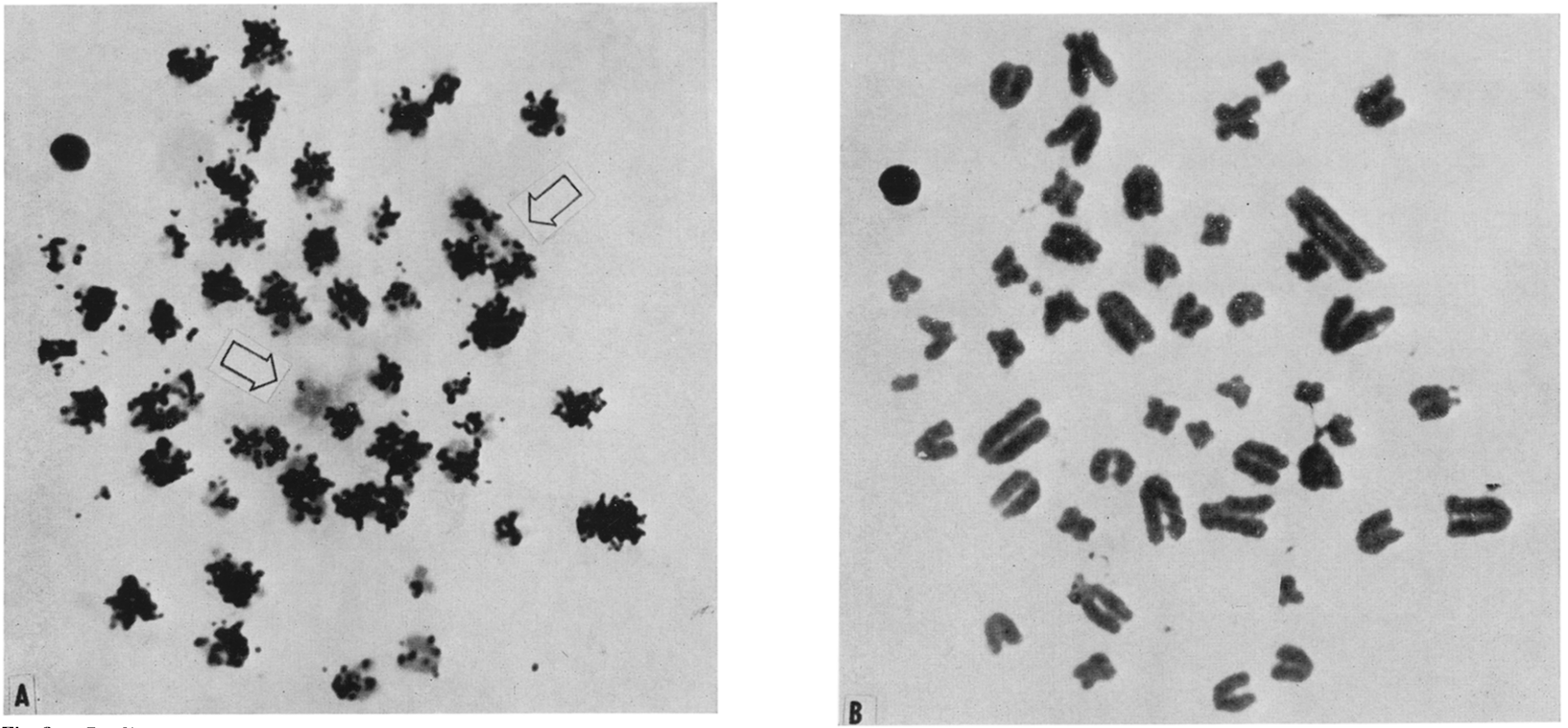


*Fig. 9. Duplicate autoradiographs of ascites metaphase (5 hrs post-labelling). The cell presents an early replication pattern, a small metacentric chromosome, unlabelled (arrow) and the chromosome (M2A) arrow) labelled only at its extremities.*

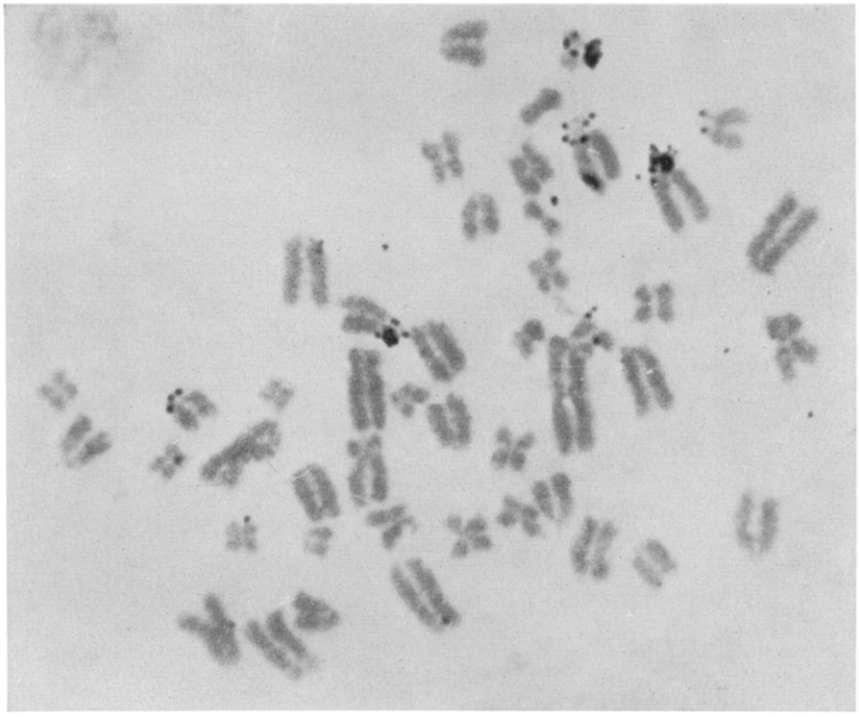

*Fig.*10. *Ascitic metaphase in an advanced stage of the phase of DNA(s) synthesis. The satellite regions and a metacentric small chromosome are late replicating.*

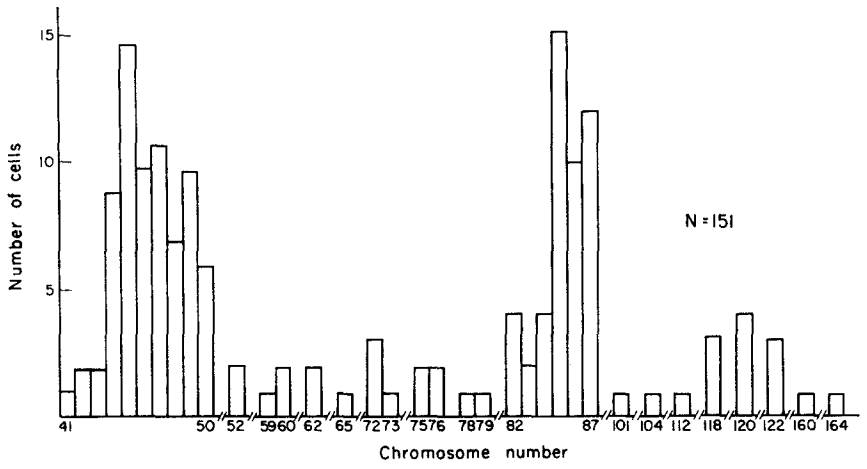


*Fig. 7. Chromosomal distribution of HR* 18 *ascites. N=number of metaphases analyzed.*

containing refringent vacuoles of lipid nature in the cytoplasm which was dissolved by staining (Fig. 6); the vacuoles were common to most neoplastic ascites.

Very large sized cells (but not only these) often presented very large vacuoles in the cytoplasm.

The chromosome distribution obtained by absolute counting of 115 cells (Fig. 7) showed the existence of two cellular populations (having 40–45 and 85–86 chromosomes) and of some more cells with high ploidy.

The analysis of the metaphases and of 25 karyotypes revealed a new subtelocentric marker chromosome in 85% of the cells (Figs. 8 and 9), as well as the maintenance of the marker (M1B) in 40% (Fig. 8) and the disappearance of the markers (M1A) and (M1D).

After 5 hr contact with tritiated thymidine, 90% of the ascites metaphases were labelled.

In both cellular populations we encountered all labelling types.

Generally, the ascites cells with low ploidy were more emphatically labelled.

Despite the disappearance of the marker (M1A) in other chromosomes, secondary constrictions were identified (Fig. 8). The labelling of these regions was strong in the cells with late replication pattern and free cf labelling in the cells with early replication pattern.

The satellite regions and some metacentric chromosomes frequently underwent late replication (Fig. 8). The marker (M2A), in most cells, presented grains at its extremities, only (Figs. 8 and 9).

Generally, a replication pattern specific to rat was identified.

No particular labelling pattern could be established in the cells belonging to the two modal classes.

## DISCUSSION

The analysis of the results has led to 5 findings:

(1) The HR 18 is a characteristic rat tumour with a chromosomally uniform population.

The cellular uniformity was the result either of a unicellular origin [5] or of a tumoural selection which led to the maintaining of the most favourable cytogenetic variant [6–8] having increased proliferative capacity.

This could account for the aggressive character of the HR 18 tumour, because the tumours with uniform population are "uniform in growth but more virulent as well" [9, 10].

(2) When heterotransplanted in AKR mice, the HR 18 tumour retained its chromosome constitution as in many other cases previously described [11–18], exhibiting an invading character as well.

(3) As in other cases [19–22], no cytogenetic differences existed between the lung metastases and the parental tumours; the lung metastatic foci were populated with cells of the parental stem-line tumour cells.

(4) The chromosome constitution of the ascites form differed of that of solid parental tumours and metastases. As other ascites [21, 23, 24], the ascites analyzed presented two modal classes and new markers.

The influence exerted by the medium of tumoural growth on the chromosome structure is reconfirmed in this case too.

(5) The late replication pattern of chromosomes of the ascites cells was specific to rat [25, 26] which emphasized the nature of HR 18 tumour as a rat tumour.

Regions with secondary constrictions, satellites and some metacentric chromosomes pre-

sented late replication. In other cells, regions with late replication on sex chromosomes and autosomes present in normal cells were altered.

Direct or indirect interaction of chemical carcinogen or virus with heterochromatin was suggested and associated with the malignant transformation of the cells [27, 28]. It is possible that the substance of a metabolic or viral nature should act in the same way in the present case as well.

## SUMMARY

*The HR 18 rat tumour derived from a human melanocarcinoma was propagated in solid and permanent ascites form, and heterotransplanted in AKR mice.*

*In the new host the HR 18 tumour preserved its original chromosome constitution and behaved as aggressively as in rat.*

*The karyological uniformity of the HR 18 tumour may account for the invading character of the tumour, as a selected cellular variant, with high malignant potentiality.*

*The comparative chromosome analysis of parental tumour, lung metastasis and ascites form revealed similarities between the solid tumoural forms and differences between these latter and the ascites form.*

*The tumoural growth medium influences the chromosome structure of the neoplastic cells.*

*Late replication pattern of ascites cells was characteristic to rat, some heterochromatic regions were altered, which may be correlated to the malignant transformation of the cells.*

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# Effect of Topical Cyclophosphamide, Methotrexate and Vinblastine on 9,10-Dimethyl-1,2-Benzanthracene (DMBA)—Carcinogenesis in the Hamster Cheek Pouch

I. S. LEVIJ, J. W. RWOMUSHANA* and A. POLLIACK
*Department of Pathology, Rothschild Hadassah University Hospital, Jerusalem, Israel*

THE PRODUCTION of squamous-cell carcinoma in the hamster cheek pouch using 9,10-dimethyl-1,2-benzanthracene (DMBA) is a well-established procedure [1, 2]. In the local strain of hamsters used in our laboratory, intraepithelial carcinoma appears after approximately 6 weeks of local treatment with DMBA, and after 12 weeks most animals have developed invasive squamous cell carcinomas [3].

Chemically induced squamous-cell carcinoma of the hamster cheek pouch has been used extensively as a model in studies of various aspects of carcinogenesis [4]. However, the effect of anti-tumor agents on this model has received little attention. Shklar *et al.* [5] demonstrated that formation of hamster cheek pouch carcinoma was promoted when the animals received subcutaneous injections of methotrexate during topical application of DMBA. Topical and subcutaneous cortisone promoted formation of hamster cheek pouch carcinoma [6–8], and topical 5-fluorouracil did not result in evident inhibition of this tumor [9].

The present paper reports the effect of topical administration of 3 different cytostatic drugs on DMBA carcinogenesis in the hamster cheek pouch. In the first series of experiments, the cytostatic drugs were administered during the period of carcinogenesis to study the effect of these substances on the process of tumor formation. In the second part, tumors were induced by application of DMBA, and these tumors were subsequently treated topically with cytostatic drugs to study their therapeutic effect. The drugs were administered topically, since it was hoped that in this way a high concentration would be obtained in the target area while the risk of generalized side effects of cytostatic treatment would be reduced.

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## MATERIAL AND METHODS

*Drugs*

The carcinogen DMBA, and the cytostatic agents cyclophosphamide, methotrexate sodium, and vinblastine sulfate, were dissolved in liquid paraffin with a specific gravity of approximately 1·0. The concentrations used were DMBA 0·5%, cyclophosphamide 1·2%, methotrexate 0·05%, and vinblastine 0·0225%. All solutions were prepared on a weight-to-volume basis. Solutions of cyclophosphamide were prepared immediately before use, those of methotrexate once a week, of vinblastine every 3 weeks, and of DMBA every month.

The solutions were applied to the right cheek pouches of the experimental animals, using a No. 4 Camel's hair brush. The brush was dipped into the solution, excess was allowed to drip off, and the pouch was then stroked firmly several times along its entire length. Pilot studies had shown that in this way approximately 0·2 ml of the solution was transferred to the pouch at each painting, containing respectively 1 mg DMBA, 2·4 mg cyclophosphamide, 0·1 mg methotrexate, and 0·045 mg vinblastine.

*Animals*

A total of 208 male Syrian golden hamsters of a local strain, 1·5 to 2 months old and weighing 55 to 65 g, were used. The animals were kept 3 to 4 in a cage, and fed Purine laboratory chow and tap water *ad libitum*.

*Experiment A. Simultaneous application of carcinogen and cytostatic drugs*

The experiment was divided into 2 parts:

(1) Eight groups of 7 animals each were treated as follows:

Group A-I received paintings with DMBA solution on 3 days per week (Sunday, Tuesday, and Thursday), and with liquid paraffin on 3 alternate days (Monday, Wednesday, and Friday). Groups A-II, A-III, and A-IV received DMBA as above, but on the alternate days they were treated with solutions of cyclophosphamide (Group A-II), methotrexate (Group A-III), or vinblastine (Group A-IV). Group A-V received paraffin only on 6 days per week, Groups A-VI, A-VII, and A-VIII received paraffin only on Sundays, Tuesdays, and Thursdays, and on the alternate days they were treated with solutions of cyclophosphamide (Group A-VI), methotrexate (Group A-VII), or vinblastine (Group A-VIII). All the above treatments were administered during a period of 6 weeks.

(2) Eight groups of 7 animals each were treated as in part 1, but for a period of 12 weeks. Group A-IX received DMBA and paraffin on alternating days, Group A-X received DMBA and cyclophosphamide, Group A-XI received DMBA and methotrexate, Group A-XII received DMBA and vinblastine, Group A-XIII received paraffin only, Group A-XIV received paraffin and cyclophosphamide, Group A-XV received paraffin and methotrexate, and Group A-XVI received paraffin and vinblastine.

*Experiment B. Treatment of carcinogen-induced lesions with cytostatic drugs*

The experiment was divided into 2 parts:

(1) Six groups of 6 animals each received topical DMBA 3 times per week during 6 weeks. After this period, Group B-I was sacrificed immediately. The other animals were anesthetized with ether, and the cheek pouches were examined for the presence of tumors. Over the next 6 weeks, Group B-II was left untreated, and the other animals received topical applications 3 times per week of paraffin only (Group B-III), or of solutions of cyclophosphamide (Group B-IV), methotrexate (Group B-V), or vinblastine (Group B-VI).

(2) Six groups of 10 animals each received topical DMBA 3 times per week during 12 weeks. After this treatment, Group B-VII was sacrificed immediately, and at this time the cheek pouches of all other animals were examined under ether anesthesia for the presence of tumors. Over the next 6 weeks, Group B-VIII was left untreated, and during this period the other animals received 3 topical applications per week of paraffin only (Group B-IX), or of solutions of cyclophosphamide (Group B-X), methotrexate (Group B-XI), or vinblastine (Group B-XII).

At the termination of the various treatments, the animals were sacrificed, and the cheek pouches, regional lymph nodes, and internal organs were examined macroscopically and histologically.

## RESULTS

The results of the 2 experiments will be presented separately. To avoid repetitive descriptions, definitions will be given of the cheek pouch lesions encountered most frequently in both experiments:

*Invasive squamous cell carcinoma.* Tumor showing marked cellular and nuclear pleomorphism, loss of polarity, an increased number of mitoses, dyskeratosis, and destruction of the basal membrane with invasion of tumor cells in the lamina propria.

*Intraepithelial carcinoma.* Lesion with cellular and nuclear changes as above, but with an intact basal membrane, and absence of invasive growth. Areas of intraepithelial carcinoma were often encountered in otherwise benign squamous cell papillomas, but these lesions were also found in macroscopically non-tumorous cheek-pouch mucosa.

*Benign squamous-cell papilloma.* Papillomatous tumor of squamous epithelium, with acanthosis and hyperkeratosis, with normal appearance of cells and nuclei, or occasional minimal atypia.

*Benign epithelial hyperplasia.* Diffuse or focal thickening of the pouch mucosa due to

*Table* 1. *Epithelial changes found in cheek pouches of hamsters treated topically for* 6 *or* 12 *weeks with DMBA, alternating with paraffin or with solutions of cytostatic agents*

| Group | Treatment | Invasive carcinoma | Intra-epithelial carcinoma | Benign papilloma | Benign epithelial hyperplasia |
|---|---|---|---|---|---|
| 6 weeks | | | | | |
| A-I | DMBA/paraffin | 0 | 3 | 0 | 7 |
| A-II | DMBA/cyclophosphamide | 0 | 3 | 0 | 4 |
| A-III | DMBA/methotrexate | 0 | 0 | 1 | 0 |
| A-IV | DMBA/vinblastine | 0 | 0 | 0 | 1 |
| 12 weeks | | | | | |
| A-IX | DMBA/paraffin | 5 | 2 | 0 | 7 |
| A-X | DMBA/cyclophosphamide | 0 | 5 | 0 | 4 |
| A-XI | DMBA/methotrexate | 0 | 5 | 0 | 3 |
| A-XII | DMBA/vinblastine | 0 | 0 | 2 | 4 |

All groups consisted of 7 animals. Numbers represent animals in each group showing lesions.

acanthosis and hyperkeratosis, with normal epithelial polarity and normal appearance of cells and nuclei, or occasional minimal atypia.

*Experiment A*

The results are summarized in Table 1. No spontaneous deaths occurred throughout this experiment.

*Part* 1. After 6 weeks, 3 of the animals treated with DMBA and paraffin had intraepithelial carcinomas. No intraepithelial or invasive carcinomas were found in animals receiving methotrexate or vinblastine alternating with DMBA, but no such tumor inhibition was found in the animals treated with DMBA and cyclophosphamide, 3 of which had intraepithelial carcinomas.

*Part* 2. After 12 weeks, all animals treated with DMBA and paraffin had carcinomas (Fig. 1), and these were invasive in 5 animals and intraepithelial in 2. There was no marked inhibition of tumor formation when DMBA was alternated with cyclophosphamide (Fig. 2) or methotrexate (Fig. 3), but all carcinomas found in these animals were intraepithelial. However, no malignant tumors were encountered in any of the animals treated with DMBA and vinblastine (Fig. 4).

Benign epithelial hyperplasia was present in all animals treated with DMBA and paraffin, but in only part of the animals treated with DMBA and a cytostatic agent. This difference was present after 6 as well as after 12 weeks.

After 12 weeks, all animals treated with DMBA and paraffin or with DMBA and a cytostatic drug had benign squamous cell papillomas of the forestomach, and in addition all animals treated with DMBA and paraffin had squamous-cell carcinomas of the skin of the cheeks. These forestomach- and skin-lesions probably resulted from spilling of the solutions. However, no such skin carcinomas were found in any of the animals treated with DMBA alternating with a cytostatic drug.

In the cheek pouches of the animals treated for 6 to 12 weeks with paraffin only (Groups A-V and A-XIII), or with paraffin alternating with cyclophosphamide (Groups A-II and A-X), methotrexate (Groups A-III and A-XI), or vinblastine (Groups A-IV and A-XIII), no macroscopic or histologic changes were found.

Regional lymph nodes and internal organs were examined in all animals, and no metastases were found.

*Experiment B*

*Part* 1 (See Table 2). In this part of the experiment no spontaneous deaths occurred, and all animals remained in good health throughout.

Among the animals sacrificed immediately after DMBA application for 6 weeks (Group B-I), 1 had a benign papilloma, but no carcinomas were found. No macroscopic tumors were found at examination under anesthesia in any of the animals treated with DMBA for 6 weeks. In 5 of the animals left untreated for 6 weeks after DMBA (Group B-II), and in 3 of those treated with paraffin only during a similar period (Group B-III), tumors were found at the time of sacrifice. Histologically there were 3 animals with invasive carcinomas

*Table 2. Effect of topical administration of 3 cytostatic agents during 6 weeks on hamster cheek pouch lesions induced previously with DMBA during 6 weeks*

| Group | Treatment DMBA for 6 wk. followed by: | At time of sacrifice | | | | |
|---|---|---|---|---|---|---|
| | | Macro tumors | Invasive carcinoma | Intra-epithelial carcinoma | Benign papilloma | Benign epithelial hyperplasia |
| B-I | Sacrifice | 1 | 0 | 0 | 1 | 6 |
| B-II | No treatment 6 wk. | 5 | 3 | 0 | 2 | 6 |
| B-III | Paraffin 6 wk. | 3 | 3 | 0 | 0 | 6 |
| B-IV | Cycloph. 6 wk. | 2 | 0 | 1 | 1 | 6 |
| B-V | Methotr. 6 wk. | 3 | 0 | 3 | 0 | 6 |
| B-VI | Vinblast. 6 wk. | 0 | 0 | 0 | 0 | 1 |

All groups consisted of 6 animals; numbers represent animals in each group which showed the lesions listed. At inspection under anesthesia immediately after DMBA, no macroscopic tumors were found in any of the animals of Groups B-II-VI.

and 2 with benign papillomas in Group B-II, and 3 with invasive carcinomas in Group B-III. No invasive carcinomas were found in animals treated with any of the cytostatic drugs, but intraepithelial carcinomas were found in 1 animal after cyclophosphamide and in 3 after methotrexate, and a benign papilloma was present in 1 animal after cyclophosphamide. None of the animals treated with vinblastine showed macroscopic or microscopic tumors. Benign epithelial hyperplasia was found in only 1 animal after vinblastine treatment, but in all animals of all other groups.

*Part* 2 (See Table 3). During the period of 6 weeks following a 12 week-DMBA application, 21 animals died spontaneously, and most of these showed pneumonia at autopsy. Since no exact examination of the cheek pouches was possible in most of these animals on account of cannibalism, only animals surviving until the time of sacrifice will be described.

Among 10 animals sacrificed immediately after treatment with DMBA for 12 weeks (Group B-VII), 6 showed invasive and 4 showed intraepithelial carcinomas. During examination under anesthesia of the remaining 50 animals, 46 had macroscopic pouch tumors.

All 4 animals allowed to survive for 6 weeks after DMBA for 12 weeks (Group B-VIII) had carcinomas, which were invasive in 3, and intraepithelial in 1. Similarly, among the 4 animals surviving treatment with paraffin only following DMBA (Group B-IX), 2 showed invasive and 2 showed intraepithelial carcinomas. Macroscopic tumors were found in all animals after cytostatic treatment. Invasive carcinomas were absent after cyclophosphamide (Group B-X), but present in 4 animals after methotrexate (Group B-XI) and in 3 after vinblastine (Group B-XII). Intraepithelial carcinomas were found in 7, 4, and 2 of these animals, respectively. None of the animals in

*Table 3. Effect of topical administration of 3 cytostatic drugs during 6 weeks on hamster cheek pouch lesions induced by previous treatment with DMBA during 12 weeks*

| Group | Treatment DMBA 12 wk. followed by: | After DMBA Macro tumors at examination under anesth. | Surviving animals after 6 wk. additional treatment | At time of sacrifice | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Macro tumors | Invasive carcinoma | Intra-epithelial carcinoma | Benign papilloma | Benign epithelial hyperplasia |
| B-VII | Sacrifice | — | — | 10 | 6 | 4 | 0 | 10 |
| B-VIII | No treat. 6 wk. | 10 | 4 | 4 | 3 | 1 | 0 | 4 |
| B-IX | Paraffin 6 wk. | 9 | 4 | 4 | 2 | 2 | 0 | 4 |
| B-X | Cycloph. 6 wk. | 8 | 7 | 7 | 0 | 7 | 0 | 4 |
| B-XI | Methotr. 6 wk. | 10 | 8 | 8 | 4 | 4 | 0 | 6 |
| B-XII | Vinblast. 6 wk. | 9 | 6 | 5 | 3 | 2 | 0 | 6 |

All groups consisted initially of 10 animals, and all animals survived the 12 week period of treatment with DMBA; numbers represent animals in each group which showed the lesions listed.

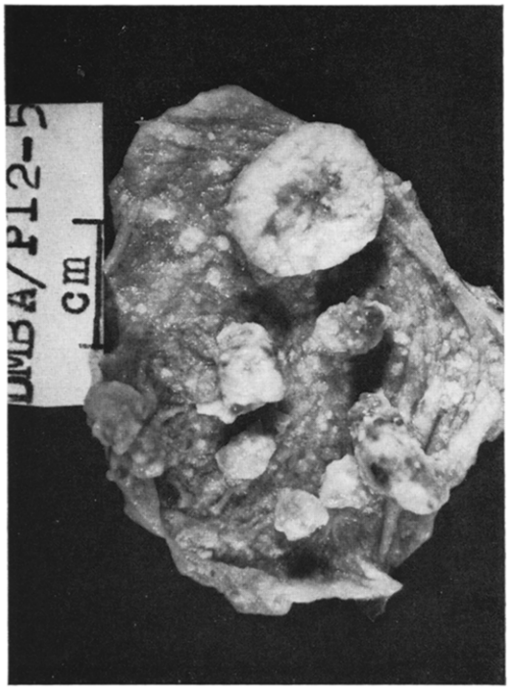


*Fig.* 1. *Hamster cheek pouch after topical treatment with DMBA alternating with paraffin for* 12 *weeks. Multiple large tumors are present, which histologically were carcinomas, partly with invasive growth.*


*Fig.* 2. *Hamster cheek pouch after topical treatment with DMBA alternating with cyclophosphamide for* 12 *weeks, showing some small tumors. Histologically these were papillomas with intraepithelial carcinoma.*

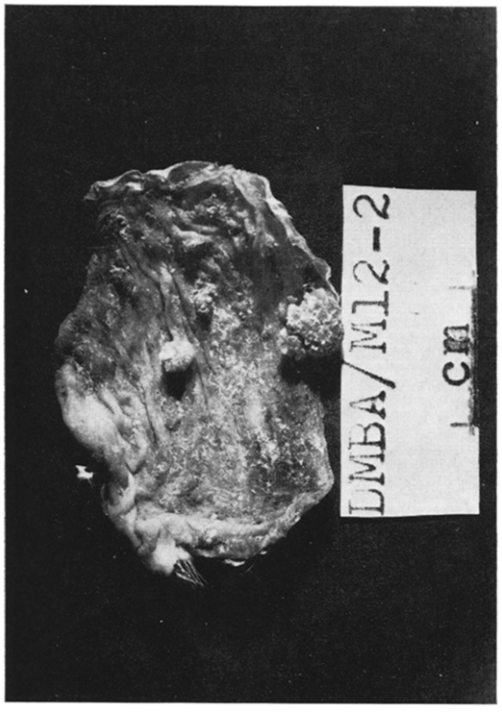


*Fig.* 3. *Hamster cheek pouch after topical treatment with DMBA alternating with methotrexate for* 12 *weeks, showing some small tumors, which histologically were papillomas with intraepithelial carcinoma.*

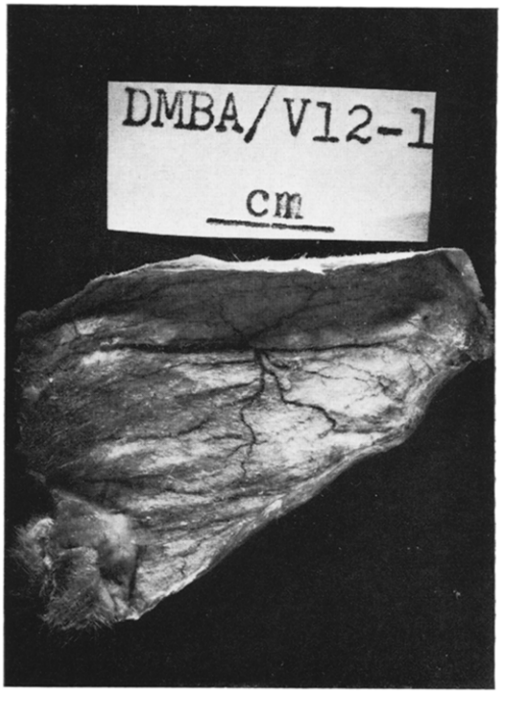


*Fig.* 4. *Hamster cheek pouch after topical treatment with DMBA alternating with vinblastine for* 12 *weeks, showing a small number of minute tumors. Histologically these were benign papillomas, and no carcinomas were found.*

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this part of the experiment had benign papillomas, since in all papillomatous tumors areas of intraepithelial carcinoma were found.

All carcinomas showed marked hyperkeratosis, and in most animals treated with cytostatic drugs partial-tumor necrosis, inflammation, and abscess formation were noted. The internal organs were normal in all surviving animals. Metastases of squamous-cell carcinoma were found in the left cervical lymph nodes in 1 animal with an invasive squamous-cell carcinoma of the right cheek pouch. This animal had been treated with vinblastine for 6 weeks after DMBA for 12 weeks (Group B-XII). Metastases were absent in all other animals.

## DISCUSSION

The drugs chosen for this study, cyclophosphamide, methotrexate, and vinblastine, are representative of 3 different groups of cytostatic drugs. Cyclophosphamide is an alkylating agent, which prevents cell multiplication by binding molecules of DNA together, thus preventing its replication [10]. This drug is inactive, but it is transformed in the liver into active compounds [11], which are accumulated selectively in neoplastic tissues [12]. Although topical application of this drug would thus seem *a priori* to be of little therapeutic value, it was hoped that a sufficient quantity would be resorbed through the highly vascular cheek pouch mucosa, and that the activated metabolites would then be redistributed to the tumor-bearing tissues.

Methotrexate is a folic acid antogonist which competes with this vitamin for folic acid reductase. Binding of methotrexate to this enzyme leads to the latter's blockage, and thus to interference with DNA synthesis [13].

Vinblastine is an alkaloid which arrests the mitotic process at the metaphase [14]. It is not known whether vinblastine acts through interference with DNA synthesis [15], or through suppression of soluble RNA, leading to depletion of proteins, including those which are essential for the development of the mitotic spindle [16, 17]. It is known that hamster tissues are remarkably sensitive to the antimitotic effect of vinblastine [18].

In the first experiment (A) presented here, complete inhibition of malignant tumor formation was noted in animals treated topically with vinblastine during DMBA carcinogenesis. Although no invasive carcinoma was found in animals treated similarly with cyclophosphamide or methotrexate, tumor inhibition with these 2 compounds was less convincing. In contrast to the findings of Shklar *et al.* [5], who used methotrexate systemically, no tumor promotion was found after topical methotrexate. This difference may be due to the higher concentration of the cytostatic agent in the target tissues, obtained in the present study.

Since infiltrative growth was not found in tumors induced in animals receiving cyclophosphamide, part of this drug was apparently resorbed through the pouch mucosa, and activated. However, it cannot be excluded that part of the cyclophosphamide applied topically spilled over to the oral cavity, and thus was in fact administered orally. An additional argument in favor of this possibility is the finding of forestomach papillomas in all animals treated for 12 weeks with DMBA with or without concurrent administration of a cytostatic agent.

Both cyclophosphamide and methotrexate interfered with tumor development, but the inhibition was incomplete. Although these drugs appear to be able to counteract the effect of a carcinogen when they are applied topically, their effect was much less marked than that of vinblastine. It may thus be concluded that of the 3 drugs studied, vinblastine is most suitable in protecting the squamous epithelium of the hamster cheek pouch against the effect of a carcinogen when administered locally. It is obvious that these findings cannot be applied directly to human pathology, since it is known that hamster tissues have an unusual sensitivity for vinblastine [18].

In part B of the study, only 1 of the 6 animals sacrificed immediately after DMBA for 6 weeks (Group B-I) had a benign papilloma, and no carcinomas were found in this group. This is in contrast to the findings in the 6 animals in part A of the study, which were sacrificed immediately after 6 weeks DMBA (Group A-I), where 3 had intraepithelial carcinomas. This difference probably resulted from variations in the induction time of hamster cheek pouch carcinoma.

Malignant tumors were absent in Group B-I, but invasive carcinomas were found in 3 animals treated with DMBA for 6 weeks followed by a treatment-free interval of 6 weeks (Group B-II), and also in 3 animals treated with DMBA for 6 weeks followed by application of paraffin only for 6 weeks (Group B-III). This shows that the induction and development of tumors apparently progressed after discontinuation of the carcinogen. Therefore the findings in animals treated with cytostatic drugs after previous administration of DMBA should be compared with those in animals where application of DMBA was followed by a treatment-free interval or by

application of paraffin only, and not with the findings in animals sacrificed immediately after DMBA.

The further development and the growth of cheek pouch lesions induced previously by application of DMBA for 6 or 12 weeks were partially inhibited when the cheek pouches were subsequently treated topically with cyclophosphamide for 6 weeks. In part A of this study, cyclophosphamide had no effect on tumor induction when applied concurrently with DMBA for 6 weeks, but during 12 weeks of this combined treatment tumor induction was slightly inhibited. It is possible that the cystostatic effect of cyclophosphamide is more pronounced in cells which have been altered previously, for example by a carcinogen, than in normal cells. This might explain why this drug had a stronger anti-tumor effect in the long-term than in the short-term study.

Methotrexate slightly inhibited the development of lesions induced by DMBA during 6 weeks, but it had no effect on lesions induced by DMBA during 12 weeks. In part A of this study, concurrent administration of DMBA and methotrexate delayed the onset of tumor formation, but the effect of methotrexate was less in the later stages of the combined treatment. It is conceivable that cells of established tumors may become increasingly resistant to methotrexate by acquiring alternate metabolic pathways, or by producing increased amounts of folic acid.

Vinblastine inhibited the development of lesions induced by DMBA during 6 weeks and caused regression of DMBA-induced benign epithelial hyperplasia. However, it had no effect on lesions induced by DMBA during 12 weeks. In 1 of the animals treated with vinblastine for 6 weeks after 12 weeks of DMBA, cervical lymph node metastases were found [19]. It is not possible to determine whether the occurrence of metastases in this animal was in any way related to the treatment with vinblastine.

The reduced therapeutic effect of the cytostatic drugs on the more advanced tumors may have been due to the relatively smaller doses applied to the larger lesions, since the same amounts of these agents were administered after 6 and after 12 weeks of DMBA. Furthermore, the progressively thickening keratin layer characteristic of this tumor may have reduced the penetration of the drugs to the underlying tumor cells.

The results seem to indicate that in the hamster cheek pouch, topical vinblastine, cyclophosphamide, and methotrexate, in that order, have some therapeutic effect on DMBA-induced precarcinomatous and early malignant lesions. However, in the dosages used, the tested drugs were ineffective in the treatment of larger more developed lesions, induced by application of the carcinogen for a longer period of time.

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## SUMMARY

*In hamster cheek pouches treated topically with DMBA 3 times per week during 6 or 12 weeks, concurrent topical application on alternate days of cyclophosphamide, methotrexate, or vinblastine, caused inhibition of tumor formation to varying degrees. Tumor inhibition was most marked with vinblastine and much less with methotrexate and cyclophosphamide. When hamster cheek pouch lesions, induced previously with DMBA during 6 weeks were subsequently treated topically with cytostatic drugs during 6 weeks, vinblastine completely suppressed the further development of DMBA-induced lesions, but topical cyclophosphamide had only a slight effect, and the effect of methotrexate was minimal. Topical cytostatic treatment of cheek pouch lesions induced by previous application of DMBA during 12 weeks had no therapeutic effect.*

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# Suppression by Spironolactone of 7,12-Dimethylbenz(a)anthracene-Induced Mammary Tumors

KALMAN KOVACS and ARPAD SOMOGYI

*Institut de Médecine et de Chirurgie Expérimentales*
*Université de Montréal, Montréal, Canada*

## INTRODUCTION

PRETREATMENT with spironolactone, a non-toxic steroid lactone, protects the rat against adrenal necrosis caused by 7,12-dimethylbenz(a) anthracene (DMBA) [1]. Since the adrenocorticolytic and the carcinogenic effects of this polycyclic hydrocarbon have been assumed to run parallel [2], it seemed worthy to investigate the action of spironolactone on DMBA-induced mammary tumors.

## METHODS

Two experiments were performed on female Sprague–Dawley rats (Holtzman Farms, Madison, Wisconsin, U.S.A.) weighing 150–180 g (approximately 50 days old) and maintained *ad libitum* on Purina Laboratory Chow (Ralston Purina Co. of Canada) and tap water.

In the *first experiment*, a single dose of 40 mg of DMBA dissolved in 2 ml corn oil was given per os through a soft rubber catheter to 70 rats, of which 20 also received spironolactone (Aldactone®, G. D. Searle & Co., Chicago, Ill., U.S.A.), at a dose of 10 mg/100 g body weight, in 1 ml distilled water by the same route twice daily for 7 days beginning 4 days prior to DMBA administration. The animals were killed with chloroform 150 days following DMBA treatment and the presence of mammary tumors was recorded when a nodule, at least the size of a small pea, could be felt.

In the *second experiment*, 80 animals were subdivided into two equal groups and received under light ether anesthesia 2 mg of DMBA in 0·4 ml oil emulsion (prepared by Dr. Paul Schurr, The Upjohn Company, Kalamazoo, Michigan, U.S.A.) into the jugular vein once daily on the lst, 4th and 7th day. Beginning 2 days prior to the first DMBA injection, 40 rats were given in addition spironolactone twice daily for 12 consecutive days, at the same dose and in the same manner as in the previous experiment. The animals were gently palpated at weekly intervals and the incidence of mammary tumors registered. One hundred and forty days after the final administration of DMBA, the rats were killed with chloroform. The tumors were excised and weighed on an analytical scale with an accuracy of 0·01 g.

In both experiments, the tumors, adrenal glands and organs showing gross abnormalities were fixed in neutral formalin and embedded in paraffin. Sections of 4–6$\mu$ thickness were cut and stained with hematoxylin-phloxine, the PAS technique and with von Kóssa's reaction for the demonstration of calcium salts. The results were evaluated statistically by the Chi-square and Student's $t$ tests.

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## RESULTS

Table 1 shows the results of the first experiment. It can be seen that mammary tumors were palpable at 150 days in all but three of the surviving rats receiving DMBA alone, whereas tumors occurred only in three rats when spironolactone was also given.

The results of the second experiment are summarized in Fig. 1 and Table 2. Within 4 weeks of DMBA administration, four rats from the first and one from the second group died and were excluded from the evaluation. Figure 1 shows that the first palpable mammary tumors appeared at about 5 weeks after DMBA treatment. In the following weeks the tumors gradually increased in size and number and all rats had at least one and, very often, more than one tumor in their breasts on termination of the experiment at 20 weeks. These findings are in general agreement with the results of Huggins and his coworkers [2–5]. However, tumor production was not as rapid in our experiments. In the group given spironolactone as well as DMBA, tumors occurred less frequently and developed at a slower rate. The Chi-square test revealed a statistically significant difference in the incidence of tumor production between the two groups.

Table 2 also shows that in rats given DMBA alone breast-tumor development was more rapid and widespread than in animals pretreated with spironolactone. The average numbers and the average weights of the tumors in the two groups differed considerably but not to a statistically significant extent when only the tumor bearing rats were compared.

The morphologic characteristics of mammary tumors developing after DMBA administration have been described by several authors [4, 6, 7]. In the present experiments, the tumors did not differ markedly from those found by others [4, 6, 7], or by us during a previous study [8]; therefore the structural features are only briefly summarized here.

The tumors became manifest along the so-called milk line, where breast tissue is normally present, with the highest incidence in the cervical and pectoral regions, but many tumors were also found at sites of the thoracic abdominal or inguinal mammary glands. Some tumors grew rapidly and attained diameters of 5–8 cm while others grew slowly and remained small. In a few animals only a single tumor was found, but the incidence in many was 2–6 tumors and, in one rat treated with DMBA alone, 13 tumors were discovered. The tumors were grossly nodular, well circumscribed, moderately firm and exulcerated in a few cases. Metastases were not observed.

Histologically, the tumors had the structure of adenocarcinoma with various degrees of differentiation (Fig. 2). Tubules and glandular formations, which were dilated in some places, were lined by a single or multiple layers of columnar or flat epithelial cells, and frequently contained acidophil secretions in their lumina. Papillary growths often projected into the lumen and in many tumors the duct-like structures were transformed into cysts. Sometimes the tumors consisted of solid cell nests; occasionally, the so-called comedo pattern was recognized. Several tumors were anaplastic with numerous mitotic figures, others showed only a slight or moderate polymorphism and polychromasia rather resembling benign adenomata. The quantity of stroma varied considerably in some places occupying a major part of individual tumors. In many instances, hemorrhages and foci of necrosis with secondary calcification were present. Inflammatory reaction also occurred frequently; the fibrous stroma was infiltrated in varying degrees by mononuclear cells and by a few polymorphonuclear leukocytes. Metastases were not observed microscopically, but invasion of the neighboring muscle tissue was common.

Besides these adenocarcinomas, the so-called milk secreting adenomas were also recognized in a few instances and, more frequently, typical fibroadenomas were seen. Four extramammary tumors were found in rats given DMBA alone; three tumors (one in the first and two in the second experiment) were squamous epitheliomas in the outer ear duct, apparently from Zymbal's gland, a sebeceous gland located periauricularly near the tympanic membrane. These tumors are known to develop in DMBA and methylcholanthrene treated rats [4, 5, 9, 10]. The fourth tumor was a cystic granulosa cell tumor in the ovary (first experiment). A typical stem cell leukemia developed in a DMBA-treated rat in the second experiment. This rat showed signs of anemia, became gradually very weak, and was sacrificed 100 days after the last intravenous injection of DMBA. At autopsy, a marked hepato- and splenomegaly was found with enlargement of the lymph nodes, anemia of the organs and petechiae on the serous membranes. Histologic examination showed that various organs were infiltrated with primitive stem cells. The liver tissue was most severely affected and, in some places, the original liver parenchyma was hardly recognizable having been replaced by a

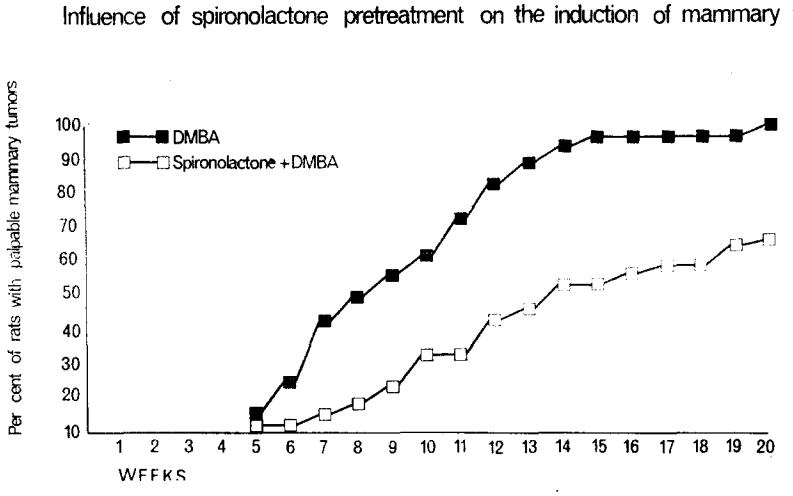


*Fig.* 1.

*Table 1. Effect of spironolactone on the incidence of DMBA-induced mammary tumors*

| | DMBA | Spironolactone + DMBA |
|---|---|---|
| Initial number of rats | 50 | 20 |
| Number of rats with palpable mammary tumors/number of rats evaluated* (at 150 days) | 21/24 | 3/14 |

*Some rats died, others were killed for other purposes before 150 days elapsed, following DMBA administration; therefore no conclusion can be drawn from mortality in the present experiment.

*Table 2. Effect of spironolactone on DMBA-induced mammary tumorigenesis*

| | DMBA | Spironolactone + DMBA |
|---|---|---|
| Initial number of rats | 40 | 40 |
| Mortality | 8 | 4 |
| Initial body weight (g) | 163±0·9† | 161±0·7 |
| Final body weight (g) | 269±4·3 | 268±4·1 |
| Number of rats with palpable mammary tumors/ number of rats evaluated | 36/36 | 26/39** |
| in % | 100 | 67 |
| Appearance of palpable mammary tumors in weeks | | |
| range | 5–20 | 5– |
| median | 8 | 12 |
| 50% | 8 | 14 |
| mean | 9·4 ±0·57 | 11·8 ±0·83* |
| Number of rats with dissected mammary tumors/ number of rats evaluated (at 20 weeks) | 32/32 | 23/36** |
| in % | 100 | 64 |
| Average number of dissected tumors | | |
| per all rats (g) | 4·6 ±0·57 | 1·9 ±0·42** |
| per tumor bearing rats (g) | 4·6 ±0·57 | 3·0 ±0·54 |
| Average weight of dissected tumors | | |
| per all rats (g) | 12·65±3·33 | 4·61±1·46* |
| per tumor bearing rats (g) | 12·65±3·33 | 7·22±2·12 |

†=standard error, *=$P<0·05$, **=$P<0·001$

difuse proliferation of atypical leukoblasts (Fig. 3).

There was no marked difference between the histologic structure of mammary tumors in the two experimental series or in the individual experimental groups. One can therefore conclude that while spironolactone pretreatment only reduced the incidence and yield of tumors it could no longer influence the degree of anaplasia once the neoplasm had developed.

In the first experiment, the adrenal glands of the rats treated with DMBA alone showed an accumulation of heavily calcified cell poor fibrous scar tissue at the corticomedullary border (Fig. 4). This characteristic lesion, which is unrelated to the production of tumors, corresponds to the ultimate phase of previously occurring DMBA-induced adrenal necrosis involving the inner layers of the cortex [11–14]. The necrotic areas were gradually replaced by granulation tissue which was transformed later into a fibrous scar [13–15]. Regeneration by the surviving cells of the zona glomerulosa resulted in the formation of a new cortex which intruded into and compressed the connective tissue. Finally, a properly zonated cortex and a well preserved medulla are usually seen with a shrunken and calcified perimedullary scar shaped like a horseshoe. These adrenal alterations, observed only in the first experiment in rats treated with DMBA alone, were absent in the animals also receiving spironolactone, which was not surprising as previous studies had shown that pretreatment with this compound protects the adrenal glands against the adrenocorticolytic effects of DMBA [1]. In the second experiment, perimedullary fibrosis and calcification were not observed. This was due to the fact that the rats were not given DMBA in sufficiently large amounts to elicit adrenocortical necrosis and, obviously therefore, the repair processes could not take place.

## DISCUSSION

The results show that, in rats, pretreatment with spironolactone, while not preventing DMBA-induced mammary tumors, significantly delays their development and decreases their incidence and yield. However, it would be misleading not to emphasize that maximal carcinogenic stimulus was applied in the present experiment and that probably a more spectacular effect might have been obtained by using smaller amounts of DMBA.

Several other experimental interventions can inhibit mammary tumorigenesis induced in rats by DMBA or 3-methylcholanthrene, e.g., bilateral ovariectomy performed before or immediately after the administration of the polycyclic hydrocarbons [15–17]. Removal of the pituitary gland or ovaries, when the tumors have already developed, arrests neoplasmic growths and caused them to regress markedly [7, 16, 18–20]. The incidence of breast tumors is also strikingly reduced in rats given a single subcutaneous injection of testosterone in their early postnatal period [8]; these androgenized animals remain permanently sterile, neither ovulating nor having any corpora lutea, and become resistant to DMBA-induced mammary cancers. Depending upon timing, dosage and experimental arrangement the administration of various hormonally active steroid compounds such as estradiol-17β [7, 17, 21], progesterone [7, 17, 21], testosterone [16, 19] and the contraceptive Enovid [22] can enhance or delay the production of breast tumors induced by polycyclic hydrocarbons. Pretreatment with various polycyclic hydrocarbons [2, 23], Sudan III [24], pituitary stalk section [18], median eminence lesions [25] and lesions in the preoptic area of the hypothalamus or the amygdaloid complex [26] also inhibit mammary tumorigenesis in rats. However, all these procedures are either toxic in short or long term experiments, or cause changes in the hormonal equilibrium and therefore their practical application appears to be remote. Spironolactone, on the other hand, is a non-toxic compound widely used in clinical medicine as an antimineralocorticoid agent which probably can be administered for long periods without any harmful consequences.

The exact mechanism whereby spironolactone delays mammary cancer development in DMBA-treated rats is not clearly understood. However, some recent findings suggest that this inhibitory action may be due to the induction or stimulation of drug metabolizing microsomal enzymes in the hepatocytes which transform the polycyclic hydrocarbon into inactive derivatives. Several observations support this view. It has been shown that spironolactone is capable of counteracting the toxic effects of various drugs (digitoxin, diphenylhydantoin, indomethacin, etc.) which are known to be influenced by intrahepatic microsomal enzymes [27, 28]. Electronmicroscopic studies also revealed [29] that spironolactone causes a proliferation of the smooth surfaced endoplasmic reticulum in the hepatocytes, an alteration which, according to many investigators [30–32], seems to be the ultrastructural equivalent of microsomal enzyme

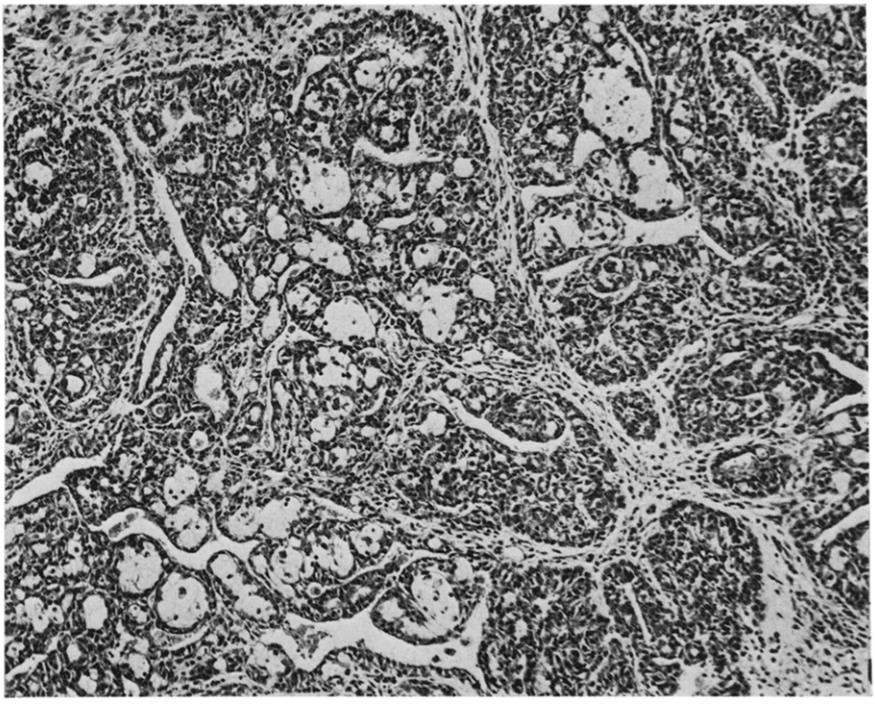

*Fig. 2. Breast tumor of a DMBA-treated rat showing the typical histological features. Hematoxylin-phloxine.* ×120.

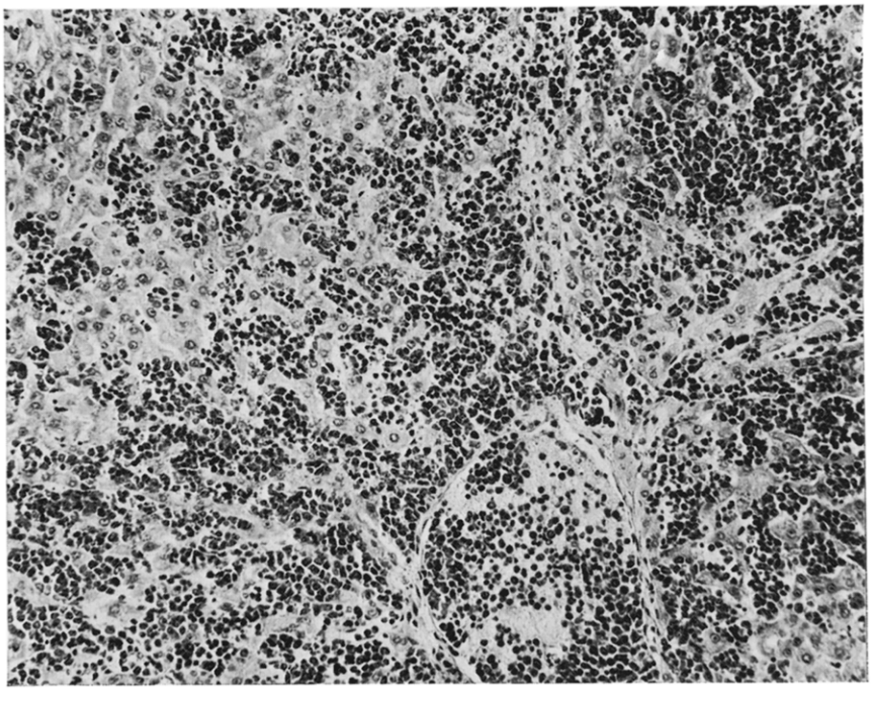

*Fig. 3. Liver of a DMBA-treated rat. The liver parenchyma is markedly replaced by proliferation of atypical leukoblasts. Hematoxylin-phloxine.* ×120.

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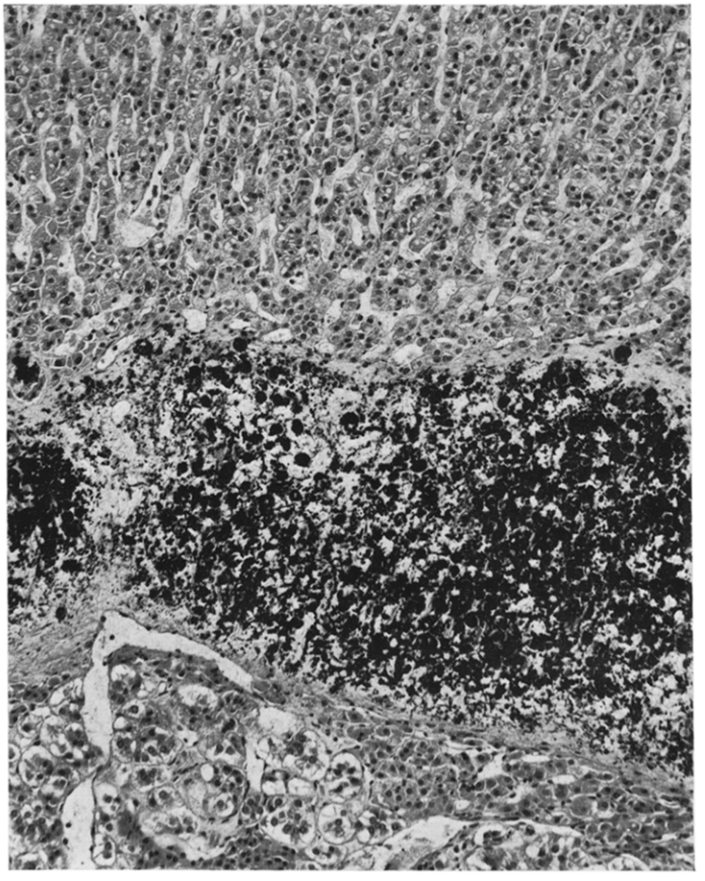

*Fig.* 4. *Adrenal gland of a DMBA-treated rat. Perimedullar fibrosis and calcification. von Kôssa reaction. Hematoxylin-phloxine.* × 120.

induction in the liver. It was also demonstrated that liver homogenates of spironolactone-pretreated rats have an increased ability to metabolize pentobarbital and that the disappearance of pentobarbital from the blood is greatly enhanced in spironolactone-pretreated rats [33]. In a previous study [34], we also found that the simultaneous administration of dl-ethionine, an inhibitor of protein synthesis, known to suppress various microsomal enzyme-dependent reactions [30, 35], abolishes the protective effect of spironolactone against adrenocorticolysis induced by DMBA. It is also known that DMBA is principally metabolized in the liver [36] and furthermore that various microsomal enzyme inducers such as polycyclic hydrocarbons, by enhancing the detoxification of DMBA, are capable of preventing adrenal necrosis and delaying the development of mammary tumors [2, 23].

Although all these data support the view that spironolactone retards DMBA-induced mammary tumorigenesis by the induction or stimulation of microsomal enzymes in the hepatocytes, direct evidence is still lacking. Therefore it would be desirable to study the intermediary metabolism of DMBA following spironolactone administration.

The concept of microsomal enzyme induction [30, 37] has greatly influenced tumor research in the last two decades. Many carcinogens have been shown to be inactivated via this mechanism, for example, liver tumors developing after the administration of aminoazo-dyes or 2-acetylaminofluorene are inhibited in rats pretreated with polycyclic hydrocarbons [38]; breast tumors caused by DMBA are also prevented by pretreatment with various microsomal enzyme inducers [2, 23]; and recently it was observed that phenobarbital pretreatment retards tumor formation induced in the liver by dimethylnitrosamine [39].

The question whether spironolactone treatment has any clinical application in the prophylaxis of breast cancer cannot be answered in the affirmative at present. It is not clear what carcinogenic stimuli are involved in the induction of mammary tumors. Neither is it known if, or to what extent, they would be inactivated by the administration of spironolactone. It is also doubtful whether one can extrapolate from studies performed on animals to human pathology. However, the idea of influencing the development of cancer by inactivating the carcinogens with microsomal enzyme-inducing compounds has already been put forward by several authors [40–42]. It was not generally accepted because, unlike spironolactone, the hitherto known enzyme inducers had serious side effects and appeared to be toxic in long-term experiments. To this extent, spironolactone appears to be promising.

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## SUMMARY

*Pretreatment with spironolactone, an antimineralocorticoid steroid lactone, delays the development and decreases the incidence and yield of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats. The mechanism of this suppressive action is not fully understood but on the basis of some recent findings it seems reasonable to suppose that spironolactone is capable of influencing the intermediary metabolism of DMBA by inducing or stimulating microsomal enzymes in the hepatocytes which convert the carcinogen into inactive derivatives. Direct evidence is still lacking but the problem warrants further study primarily because spironolactone, in contrast to the other microsomal enzyme inducers, is a non-toxic compound which can be administered for a considerable length of time without serious consequences.*

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# Comparison of Cell Ultrastructure of an Experimental Ascites Tumour of the Mouse at Different Ages after Transplantation

E. DELAIN* and E. FRINDEL†

**Laboratoire de Microscopie Electronique and †Unité de Radiobiologie Clinique INSERM (U-66) Institut Gustave-Roussy, (94) Villejuif, France*

## INTRODUCTION

IT HAS been noted, in numerous transplanted tumours, that growth is rapid at first and then slows down as the tumours get older [1, 2]. We have found the same deceleration of growth, as a function of age, in the NCTC fibrosarcoma growing *in vitro*, as a solid tumour [3] and in the ascites form [4].

*In vitro*, and in the solid form, we have found that though the doubling time increases exponentially with age, the cell cycle seems to remain constant while the growth fraction (G.F.) diminishes. In the ascites form of the tumour, the doubling time also increases with age as does the cell cycle time and the various phases of the cycle, while the G.F. remains constant. The deceleration of growth may be due to various factors and it has been our interest to try to elucidate the mechanism of this phenomenon.

In a previous study, we were able to demonstrate that neither the oxygen tension nor the lack of glucose could explain the increase of cell cycle times [5]. Immunological factors do not seem to play a role as the phenomenon has been found *in vitro* [3].

It was of interest to explore the fine structure of the young and old cells in order to verify if cellular organelles deteriorate during the aging of the tumour.

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## MATERIAL AND METHODS

*The cells*

The fluid form of the NCTC fibrosarcoma was obtained in our laboratory [4] by injecting intraperitoneally NCTC 2472 cells cultured *in vitro* [6]. During the first few passages on isologous $C_3H$ female mice, solid-tumour formation was observed and only small amounts of fluid were present. The pure ascites form was obtained after about twenty-five weekly passages. The growth curve and other characteristics of this tumour are reported elsewhere [7].

*Electron microscopy*

Glutaraldehyde is added to the ascites cell suspension to obtain a final concentration of 1·5%. The cells are pelleted for 2 min at 4000 rev/min the supernatant discarded and replaced by fresh and cold glutaraldehyde in Sörensen phosphate buffer at pH 7·2. The cells are resuspended in the fixative and stored at +4°C for 1 hr. After a 10 min centrifugation, the pellet of cells is cut into small pieces and washed for 1 or 2 hr in the Sörensen buffer. The material is then post-fixed in 1% osmium tetroxyde in the same buffer, dehydrated in ethanol and embedded in Epon [8]. The sections are contrasted firstly 2 or 20 min in 2% uranyl acetate in Michaelis buffer at pH 5 and then 2 or 10 min in lead citrate, according to the method of Reynolds [9]. The sections are covered with vacuum-evaporated carbon,

and observed in a Philips EM 300 electron microscope.

*Results*

Figure 1 shows the general aspect of the 4 *and* 5-*day old tumours* (Plate I). The large nucleus (N) is often irregular in shape and possesses one or more nucleoli (Nu). The cytoplasm is rather dense and the plasma membrane shows a great number of microvilli attesting cellular activity. In the elongated-shaped mitochondria (M), the cristae are clearly visible. In different cells, the mitochondria are more or less abundant. They are often surrounded by the granular endoplasmic reticulum (E). Besides, a large number of free cytoplasmic ribosomes (R) may be found in some cytoplasmic areas without any other organelles. The Golgi complex region (G) takes up a considerable space and it can be noted that occasionally there are some intracisternal A-type virus particles (small arrows), which are present in the vesicles of the smooth-surfaced endoplasmic recticulum, at the neighbourhood of the Golgi zone. Occasionally intravacuolar C-type virus particles can be seen on the outer surface of the cell or in an intracytoplasmic vacuole (Fig. 1, large arrow).

In Fig. 2, the cell membrane exhibits some micropinocytosis vesicles in formation (arrows), illustrating the absorption of substances from the ascitic fluid. In the adjacent, free ribosome-rich cytoplasm, numerous mitochondria (M) and rough-surfaced ergastoplasmic lamellae (E) are seen.

Figure 3 shows a portion of the Golgi zone (G); in its vesicles are two budding A-type virus particles (v). The peripheral cytoplasm contains mitochondria (M) and many free ribosomes (R). The endoplasmic reticulum is abundant and, at some places, swollen by granular material. Such a picture suggests a high metabolic activity of the cell. Discrete lipid droplets (L) may be encountered.

Plates II and III show the ultrastructure of the older ascites cells (14-*day old tumours*).

There is no striking morphological modification between the 2 and 14-day old cells. In most cases, it is impossible to distinguish between the two batches of cells by their morphology. In the oldest cells (Fig. 4) the nucleus (N) is large and often contains a voluminous nucleolus, which is not seen on this photograph. However, in few old cells, some modifications may be observed: the mitochondria (M) have irregular shaped cristae and their general profile is rather circular. The granular endoplasmic reticulum (E) is normal, but the free cytoplasmic ribosomes (R) are not very numerous. Some lipid droplets (L) are frequent in the cytoplasm.

Figure 5 shows the activity of the cell membrane which produces microvilli and micropinocytosis vesicles (arrows). The cytoplasm is quite similar to the cytoplasm of the young cell shown in Fig. 1.

In Fig. 6, the mitochondria exhibit the irregular shape of their cristae and their rounded profile. The free ribosomes (R) are still abundant in the older cells. Note the two lipid droplets (L).

Some of the 14-day old cells are modified to a greater extent than those shown in Figs. 4, 5 and 6. Figure 7 illustrates such a degeneration. The cell may contain two or three nuclei (N); these nuclei are often pycnotic and have numerous cytoplasmic invaginations. In the cytoplasm, the rough-surfaced ergastoplasm and free ribosomes are present, but the mitochondria have been reduced in number and size (M). The lipid vacuoles however cover a very important volume in the cell and in such cells the microvilli are rare, and micropinocytosis is absent.

## DISCUSSION

It seems, from ultrastructural studies of the young and old tumours, that the cells are practically unmodified with age. If we exclude some degenerated cells that are encountered in the older groups, the cells exhibit ultrastructural components which seem to be "functional". We also noted no differences between the 4 and 14-day old cells, with respect to presence of the A and C-type virus particles, which are often encountered in murine tumour cells (reviewed in 10).

The fact that micropinocytosis exists in older cells seems to imply that these cells continue to absorb material from the ascites fluid. Likewise, the constant presence of the Golgi apparatus is evidence of the metabolic activities of the cells in 14-day old tumours as well as in 4-day old tumours.

The existence of mitochondria in similar quantities in young and old cells confirms our previous results that aerobic respiration continues in the older tumours. However, some alterations in the mitochondria in the oldest cells could explain a reduction of activity.

The presence of polyribosomes on the endoplasmic reticulum membranes indicates that the protein synthesis should be possible; however, it does not signify that there is no qualitative or quantitative difference between

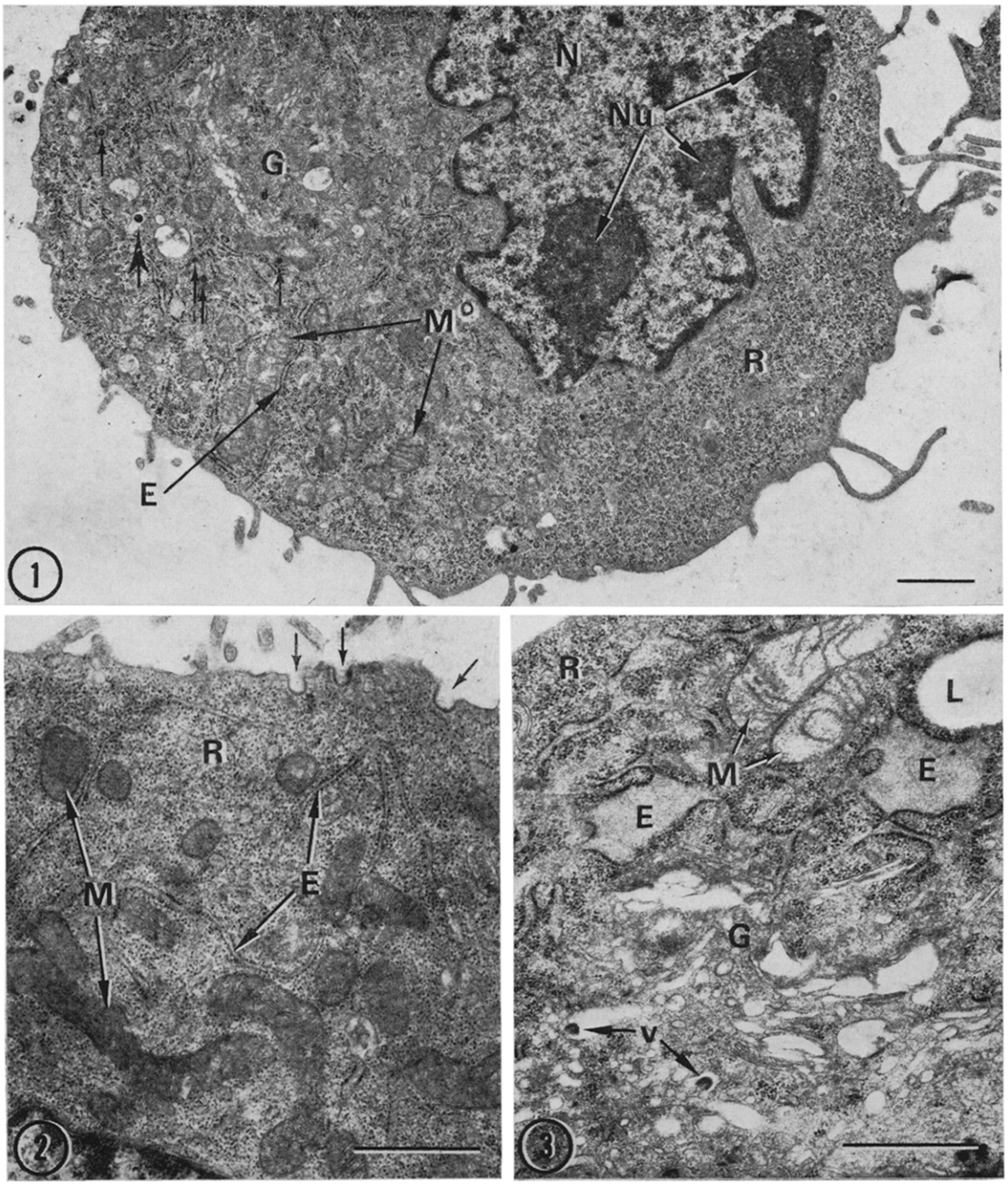


*PLATE I: Fig.* 1. *Ascitic cell,* 4 *days old, showing the general morphology of the young cells. The nucleus is large and distorted. Several nucleoli are often present. In some wide cytoplasmic areas, the free ribosomes are numerous. The Golgi apparatus is surrounded by mitochondria and endoplasmic reticulum. In smooth-surfaced vesicles, A-type virus particles are present (small arrows). Infrequently C-type virus particles may be observed in cytoplasmic vacuoles. The plasma cell is composed of many microvilli. E: Endoplasmic reticulum; G: Golgi zone; L: lipid droplet; M: mitochondria; N: nucleus; Nu: nucleolus; R: free cytoplasmic ribosomes. The scales represent one micron.*

*Fig.* 2. *Ascitic cell, 5 days old, showing the abundance of mitochondria, ergastoplasmic lamellae and free ribosomes. Note the presence of micropinocytosis vesicles (arrows) and the microvilli on the plasma membrane.*

*Fig.* 3. *Ascitic cell,* 4 *days old, the lower part of the photograph showing a Golgi zone with two budding A-type virus particles. The upper part is rich in free ribosomes and contains mitochondria and swollen ergastoplasmic cisternae. The lipid droplets are rare.*

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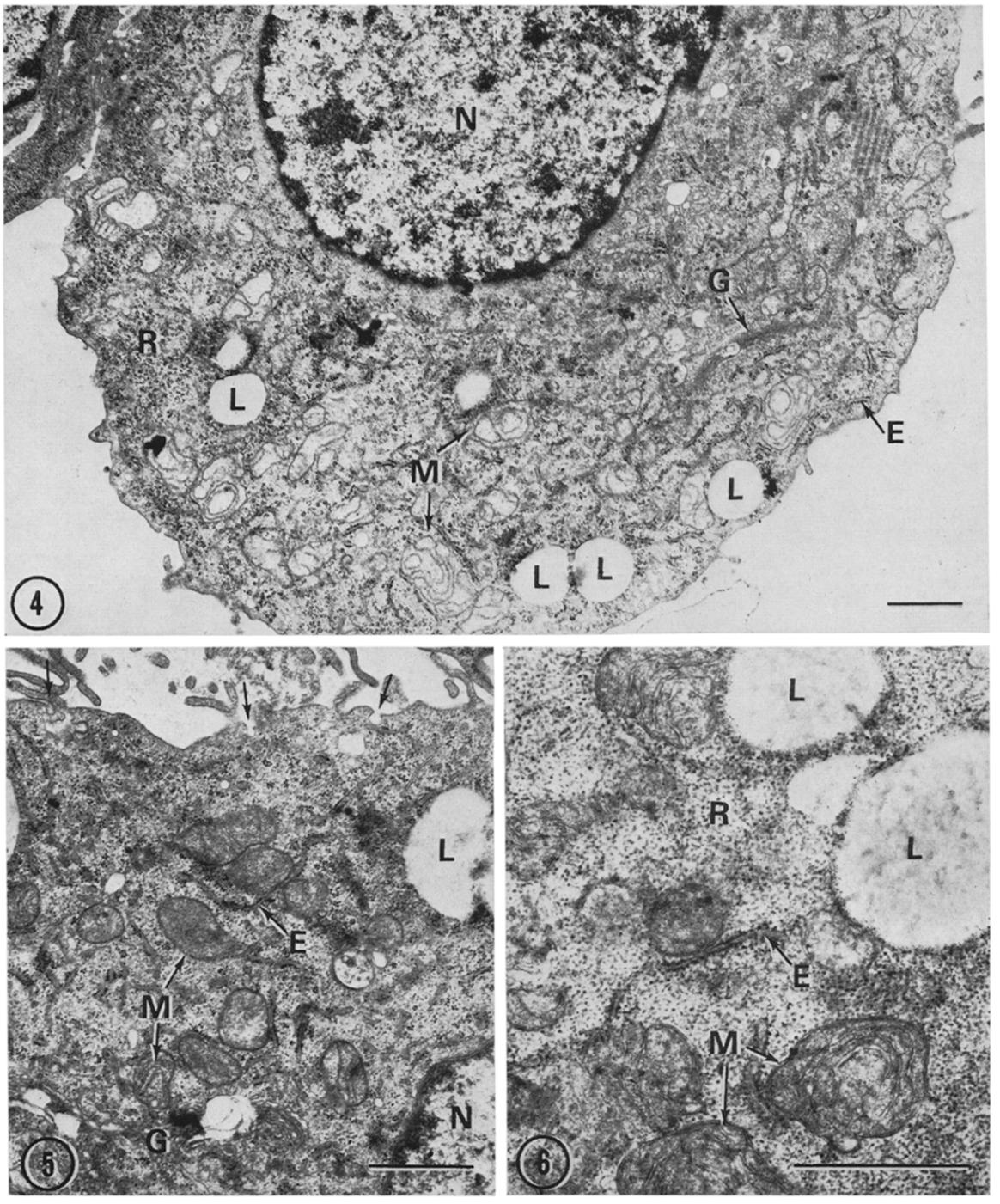


*PLATE II: Fig.* 4. *Ascitic cell,* 14 *days old, showing the general morphology of the old cells. The nucleus is large and round. The cytoplasm contains well developed Golgi apparatus and endoplasmic reticulum. The free ribosomes are not very numerous. The mitochondria are slightly swollen and have irregular cristae. The lipid droplets are rather small but are always present.*

*Fig.* 5. *Ascitic cell,* 14 *days old. The Golgi zone, the ergastoplasm and the mitochondria are normal in the cytoplasm. The free ribosomes are in small number. Note the presence of micropinocytosis vesicles (arrows) and the microvilli on the plasma membrane. A lipid droplet can be seen on the right.*

*Fig.* 6. *Ascitic cell,* 14 *days old. Among numerous free ribosomes, we can see lipid droplets, ergastoplasmic lamellae and round-shaped mitochondria, with irregular cristae.*

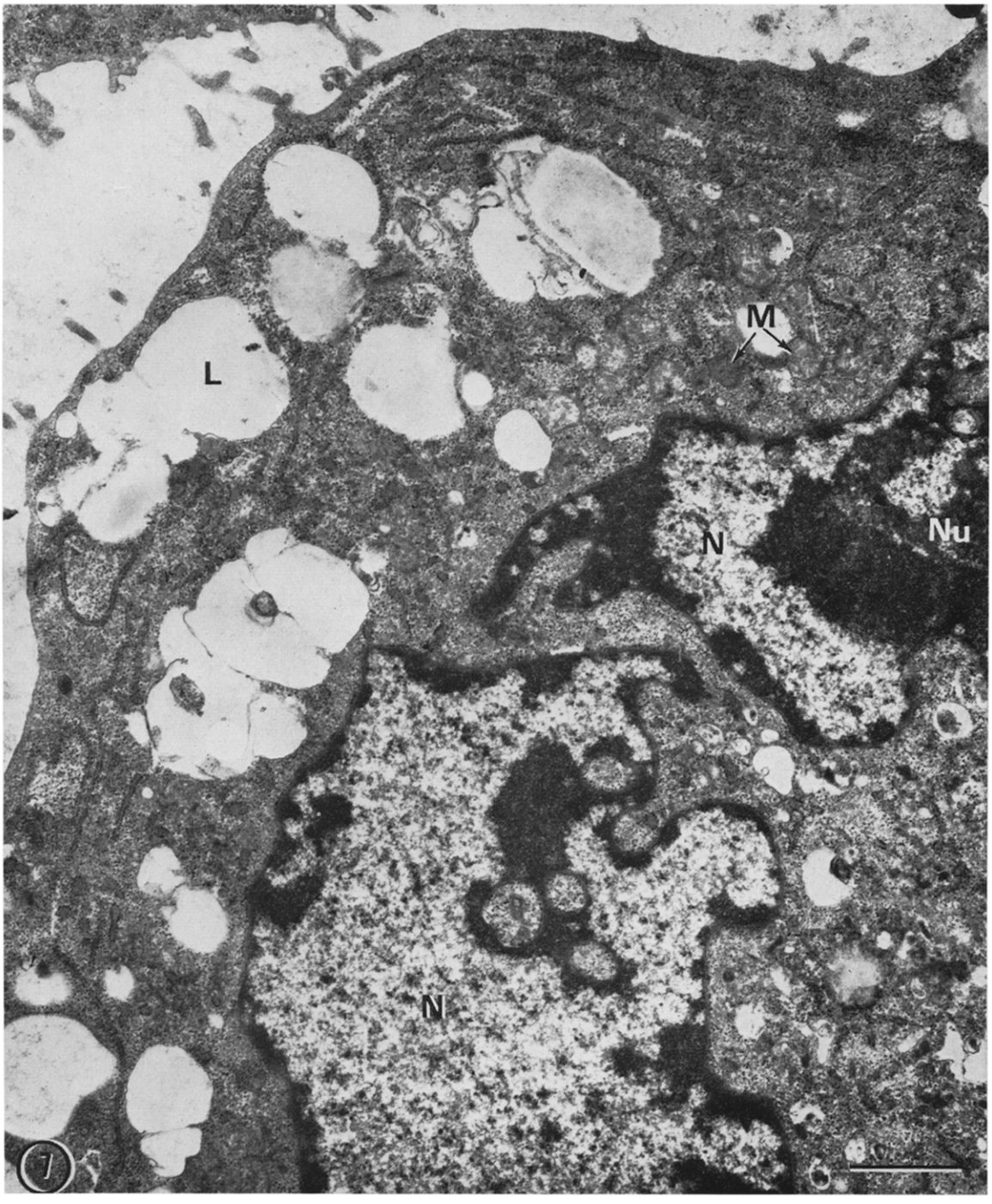


*PLATE III: Fig. 7. Ascitic cell, 14 days old. The cells may be severely damaged. They may have several irregular shaped nuclei. The cytoplasm contains only few mitochondria, a reduced ergastoplasm and free ribosomes. The lipid inclusions are then very large and conspicuous. The activity of the cell membrane is poor: indeed few microvilli and no micropinocytosis can be seen.*

the two tumours. Likewise, the lack of differences between the nuclei of the old and young cells does not necessarily indicate its normal activity. The presence of the cellular organelles does not exclude the possibility of an inhibitor that may modify the function of these organelles even if they seem normal morphologically.

In conclusion one may say that there is no visible deterioration of cellular organelles with age that could explain the deceleration of growth in the NCTC fibrosarcoma in its fluid form. The few differences in the morphology of mitochondria and in the number of free ribosomes and lipid droplets do not seem sufficient to account for the observed differences in growth rate of young and old tumours.

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## SUMMARY

*The ascitic form of the NCTC fibrosarcoma of the C3H mouse grows rapidly at first and then slows down as the tumour gets older. Electron microscopic investigations have been performed on 4-day and 14-day old cells to compare their ultrastructure. Except for some degenerating cells, the cells are practically unmodified with age. No significant differences have been found between the two tumours for the nucleus morphology and for the quantity of mitochondria and ribosomes and for the Golgi apparatus. However, the mitochondria seem to be somewhat altered in the oldest cells.*

*The few morphologic alterations observed in cytoplasmic organelles seem insufficient to explain the decelaration of growth in the NCTC fibrosarcoma in its fluid form.*

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# Time-Dose Relationships in Radiotherapy

J. F. FOWLER*, R. H. THOMLINSON†, A. E. HOWES*

**Medical Physics Department, Royal Postgraduate Medical School, Hammersmith Hospital, London, W.12;*
*and †M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W.12*

## 1. INTRODUCTION

ABOUT half the new patients with cancer are treated by radiotherapy. In some cases radiotherapy and surgery give a greater probability of cure than either method alone. Radiation and chemotherapy may also be combined. Even small improvements in the effectiveness of radiotherapy therefore benefit large numbers of patients. It is necessary to emphasize this, since the law of diminishing return is sometimes mentioned as a reason for putting less effort than before into this form of therapy; radiotherapy is already reasonably good for curing primary tumours in certain sites.

There are two main ways in which it has been said that radiotherapy might be improved: we shall see that these are inseparable. The first is by the rational use of dose fractions with appropriate intervals, and the second is by overcoming the problem of hypoxic (and therefore radiation-resistant) cells in tumours. In order to deal with the problem of hypoxic cells in tumours, high presssure oxygen tanks or fast neutron beams are being tried, and negative $\pi$ mesons have been proposed. We shall discuss in this paper how appropriate doses and intervals might in part overcome the problem.

## 2. NORMAL TISSUE INJURY

At present, radiotherapists generally give doses which are limited only by the normal tissue reactions rather than by the expected response of the tumour. In order to give radiotherapy a rational biological basis, a knowledge of normal tissue reactions for various regimes of treatment is therefore essential.

### 1. *Short-term repair*

No optimal fractionation schedule may as yet be proposed. Nevertheless, total doses producing the same reactions in certain types of normal tissue can be proposed for various changes in the number of fractions and overall time of treatment. This is one of the few examples where radiobiology is able to make a definite contribution to radiotherapeutic practice. We may say quite definitely that, should a smaller number of fractions be given in the same overall time, the total dose required to achieve similar biological effects in certain types of normal tissues must be reduced, and vice versa. The main explanation of the increase in total dose with increase in number of "daily" fractions appears to be the rapid recovery from sub-lethal injury in irradiated mammalian cells [1]. Du Sault [2] had earlier speculated that short-term repair might occur and some early work on skin-reactions had measured it unknowingly [3].

Most mammalian cells respond to increasing doses of radiation by a reduction in the proportion of cells capable of proliferating indefinitely. If this proportion is plotted on a logarithmic ordinate against the dose on a linear abscissa, the resulting cell survival curves are shaped as in Fig. 1 with an "inefficient" (shoulder) region at the low doses. Elkind and

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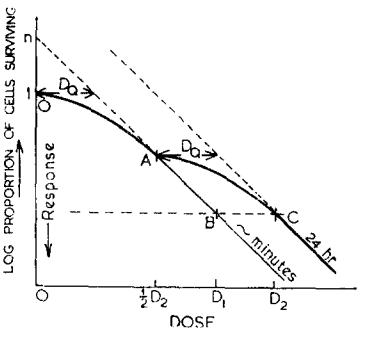


*Fig. 1. Schematic representation of the dose increment* $D_Q$ *which is required for each additional dose fraction in the first-order iso-survival theory. A single dose* $D_1$*, given within a few minutes, reduces cell survival to B. A smaller fraction* $\frac{1}{2}D_2$ *reduces survival to A, and then if an interval of 24 hr elapses before another fraction of* $\frac{1}{2}D_2$ *is given, the total dose* $D_2$ *in two fractions gives C. This is the same level of survival as B, but* $D_2$ *is greater than* $D_1$ *by the dose increment* $D_Q$ [26].

Sutton [4] found that for Chinese-hamster cells *in vitro* the inefficient shoulder of the cell survival curve reappeared within a few hours after a single dose (Fig. 1). Therefore the first one to two hundred rads of a subsequent dose given 12–16 hr later was less efficient in terms of cell killing, coincident with the reappearance of the shoulder of the cell survival curve.

Hence, each additional fraction required that a certain "wasted dose" or "dose increment" should be added to the total dose needed to produce the same level of cell survival (Fig. 1). This dose increment was called "$D_Q$" (quasi-threshold) by Alper *et al.* [5] with the application to fractionated radiotherapy in mind. $D_Q$ is represented by the intercept between the abscissa drawn through 100% cell survival and its intersection of the straight part of the logarithmic plot of cell survival against dose. If $D_2$ is the total dose in two fractions required to produce the same damage as a single dose $D_1$, then the experimental dose increment is $D_2-D_1$, which in Fig. 1 is equal to $D_Q$.

The concept that each additional fraction after the first requires a relatively constant increase, $D_Q$, to be added to the total dose constitutes the "first-order iso-survival theory" described by Elkind [1] and Elkind *et al.* [6]. It has had a great impact on thinking and research in radiobiology applied to radiotherapy. It suggests that each additional dose fraction requires *the same* increment of extra dose (which is about 100 to 200 rads for most mammalian cell lines which have been studied) to compensate for the injury repaired by intracellular processes. However, if each dose were too small to be beyond the shoulder of the survival curve, the dose increments would be correspondingly smaller.

It is now known that the dose increment in fractionated doses is not *exactly* equal to $D_Q$; the observed values differ as follows. Each successive dose is applied to the cells which survive the previous dose. If these are more resistant than the average of the heterogeneous population [7], the shape of their survival curve would be different from that derived from single doses and $D_2-D_1$ will be greater than $D_Q$, since $D_Q$ is taken from the survival curve for single doses. Because these relatively resistant cells are selected from a limited phase of the cell cycle, the survivors will progress through the ensuing cycle in partial synchrony. After a few hours of delay of division, the surviving cells pass first into a phase more sensitive than the average and after approximately one cell-cycle time pass again into a relatively resistant phase. Intracellular recovery of sublethal injury occurs within the first hour after each dose [6]. In addition, any cell division occurring between doses would increase still further the dose required to produce a given level of cell survival. Calculations including both these effects were carried out by Young and Fowler [8] using Sinclair's data for Chinese hamster cells *in vitro* [7]. It was found that the dose increment based on a $D_Q$ of 260 rads, as expected from the simple theory, would be increased to a $D_2-D_1$ of 360 rads after a first dose of 750 rads (Fig. 2). Sinclair has published an experimental result which agrees closely with this calculation (Fig. 2).

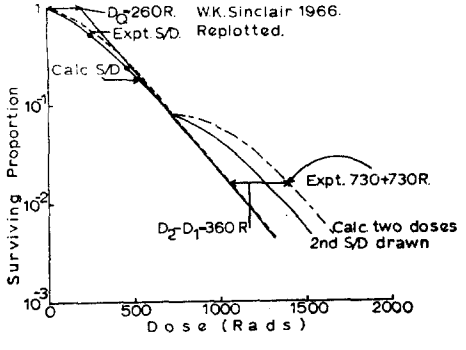


*Fig. 2. The difference between "*$D_Q$*" and "*$D_2-D_1$*" in practice. The dotted line "Expt S/D" shows Sinclair's experimental cell survival curve. The full line "Calc S/D" shows the theoretical reconstruction of a survival curve using data from different phases of the cell cycle. The chain dotted curve "Calc two doses" shows the survival curve followed by second doses given 24 hr after 750 rads allowing, for induced synchrony. It is higher than the full curve "2nd S/D drawn", which is a repeat of the single-dose shoulder as in the simple theory. Sinclair's experiment with 730+730 rads agrees closely with the calculated dose increment of 360 rads predicted by allowing for cell synchrony* [8].

For first doses smaller than 730 rads, a lesser degree of synchrony should be induced, so that the difference between $D_Q$ and observed values of $D_2-D_1$ would be smaller. Since individual doses of 150–300 rads are usual in clinical practice, Elkind's model of constant amounts of repair after repeated equal-dose fractions would be expected to be a reasonable approximation. The approximation would be least good for small numbers of fractions, i.e. for large doses of about 1000 rads per fraction, where appreciable cell synchrony might be induced. Observations on animals and on human patients have shown much larger-dose increments for two, three and four fractions than would be expected from the simple theory [9].

Figure 3 shows the changes in shape of cell survival curves calculated by Young and Fowler [8] assuming the degree of induced synchrony found in Chinese-hamster cells by Sinclair. In this cell line, therefore, changes in slope of the survival curve and hence of $D_2-D_1$ are to be expected to depend on the size of the first dose and the length of the interval after which the second dose is given. Similar effects might be expected in any cell line having large changes in sensitivity with age in the cell cycle. The effect of synchrony might therefore be appreciable in experiments in which large doses are given to tumours or normal tissue. This is particularly likely to be important in experiments where only two doses are given. If four or five fractions are used, however, the effect of synchrony would probably be lessened because it is unlikely that each dose would fall in the same phase of the cell cycle. If synchrony were induced in a cell population, a fluctuation in sensitivity would be expected at varying times after a large single dose. From the evidence at present available [10, 11] the timing of such fluctuations does not however appear to be sufficiently reproducible to permit serious hopes of using them to advantage in gaining a differential effect on tumours as compared with normal tissues by appropriate spacing of fractions. Moreover, it would be harmful if a treatment dose were given in the unintended phase of sensitivity, when the normal tissue might be unexpectedly radiosensitive relative to the tumours.

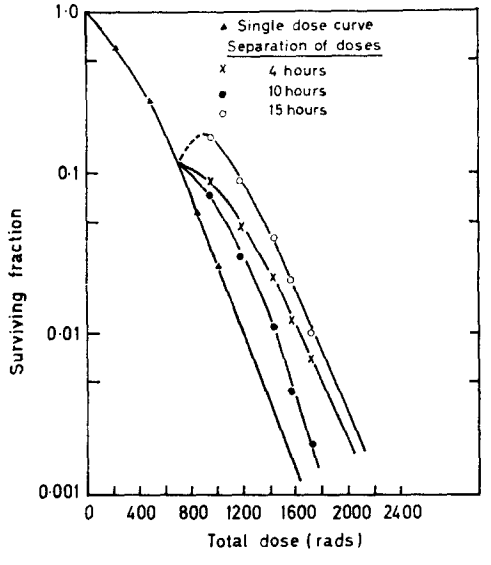


*Fig. 3. The variation in survival curve shape with time after a first dose of 750 rads. At the time of the "trough" in the recovery vs. time curve, 10 hr, the slope is the steepest but the extrapolation number high. At 4 hr and 16 hr the survival is higher although the slopes are less steep.*

2. *Repopulation*

In the "first-order iso-survival theory" of fractionation [1], repopulation must be considered separately from the effect of short-term recovery occurring after each dose fraction. It is necessary to know how large an effect cell proliferation during the whole period of treatment has on the total dose required to produce a given degree of injury to a tissue. An answer was found from experiments on the skin of pigs described below. Repopulation was found to be certainly not negligible, but was smaller than the effect of changing the number of dose fractions.

The relative effects of prompt repair of intracellular injury and of proliferation were deduced from experiments on the skin of pigs [9, 12]. Observations were based on desquamation due to depletion of the population of epithelial cells. In these experiments the epidermis, dermis and the associated vascular and nervous systems were irradiated together, using irradiation fields 5 cm × 4 cm in size as in the treatment of patients, and the injury observed might represent the response of the whole organ [13]. This point is discussed further in the next section. Table 1 shows the results of the experiments on pig skin.

The single X-ray dose required to produce a given degree of skin desquamation was found to be 2000 rads; to produce the same reaction using five fractions in four days required an

*Table 1. Iso-effect doses: Desquamation reaction in skin of pigs*

| | | |
|---|---|---|
| Single dose | 2000 rads | |
| 5 fractions/4 days | 3600 rads | } 600 rads |
| 5 fractions/27 days | 4200 rads | } |
| 21 fractions/27 days | 5600 rads | |

increase in dose of 80%, to 3600 rads. This increase provides an estimate of the four dose increments $D_Q$ necessary to overcome four sets of intracellular recovery plus any repopulation in the 4 days after the first dose. The total dose increments were therefore 1600/4= 400 rads each. When, however, five fractions were given over 27 days instead of 4 days, it was found that the dose required to produce the same skin reaction rose from 3600 to 4200 rads, a further increase of only 17%. This latter increase has been attributed by Fowler *et al.* mainly to cell proliferation occurring between 4 and 27 days in this system. The curve relating log total dose to log overall time [14] for a constant number of fractions had a log–log slope (or exponent) of 0·09 for the five fractions described in Table 1.

Figure 4 summarises results of this and other experiments on pig skin [15]. In each case where the same number of fractions was given in different overall times, a larger dose was required at the longer time. The differences

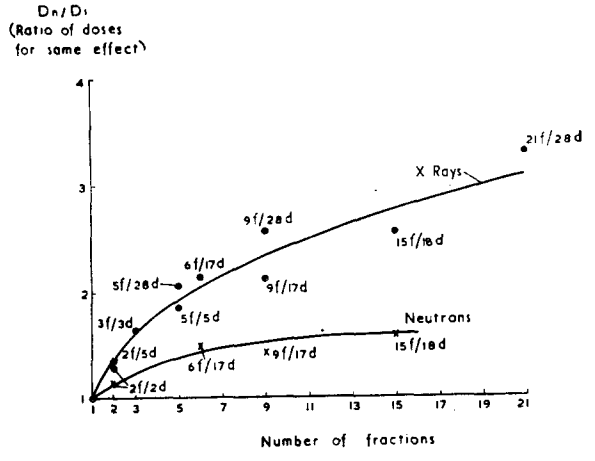


*Fig. 4. Total dose (as ratios) vs. number of fractions for pig skin desquamation. Upper curve: X-rays (2000 rads=1·0). Lower curve: cyclotron neutrons. In the X-ray curve, 5 fractions/28 days required a significantly higher dose than 5F/5d. Similarly 9F/28d required a higher dose than 9F/17d. These differences represent repopulation in the basal layer* [15].

may be used to give estimates of repopulation rate, and hence to calculate a "mean skin repopulation rate." The values obtained are not negligible, but their effects are smaller than the effect due to intracellular recovery between each fraction. The values do not differ significantly from Cohen's [16] estimate of repopulation in human skin equivalent to 25–30 rads/day averaged over several weeks. If $D_0$=150 rads*, the population doubling time would then be about 5 days. This value is in agreement with other estimates for the basal layer of human epithelium, as discussed by Fowler [17].

3. *Significance of observations of gross skin reactions*

The correlation between the time of occurrence of gross skin reactions and the predicted time of maximum depletion of cells of the epithelium (predicted from a knowledge of the population turnover times in the basal layer of the skin of pigs, mice, rats and human beings) suggests that desquamation is due to depletion of the epithelial layer caused by radiation killing of cells in the basal layer [17].

The correlation between the degree of gross skin reaction [18] and the number of clones of skin cells growing after X-irradiation [19] as determined by Withers [20] technique supports this view. Suit and Howard [21] have found that the oxygen enhancement ratio for desquamation was equal to that for epilation and also to that for contraction of skin by a specified amount in half of the irradiated thighs in C3H mice, both for single doses and for 10 fractions in 9 days (Table 2). Field, Jones and Thomlinson [22] found that the values of oxygen

*$D_0$=Dose required to reduce the proportion of surviving cells to 1/2·718 of a given value, on the straight part of the semi-logarithmetic survival curve in Fig. 1.

*Table 2*

| Suit and Howard | | Single dose | | 10 Fractions | |
|---|---|---|---|---|---|
| End-point | | Hypox/Air | Hypox/HPO | Hypox/Air | Hypox/HPO |
| DESQUAM. 21d | $DD_{50}$ | 1·2 | 1·9 | 1·6 | 2·5 |
| | $DD_{90}$ | 1·2 | 2·1 | 1·6 | 2·5 |
| EPILATION 40d | $ED_{50}$ | 1·2 | 1·9 | 1·5 | 2·5 |
| CONTRACTION 27·5 22 mm 40d | $CD_{50}$ | 1·45 | 2·15 | 1·57 | 2·47 |

enhancement ratio (OER) and relative biological effectiveness (RBE) of cyclotron neutrons in producing skin desquamation in rats at 8–30 days were the same as those for skin desquamation at 12–14 weeks and to leg deformity at 14 weeks. These findings suggest that results based on skin desquamation reactions can be correlated with those obtained using other biological end points relating to the dermis.

Other types of normal tissue damage have been studied by Bakowska, Lindop and Rotblat [23] at St. Bartholomew's Hospital, London. They found that changes in capillary diffusing permeability soon after irradiation of mouse ear were *not* correlated with the dose-sparing effects of short-term prepair or repopulation, and the significance of the changes in permeability are therefore not known. However, the appearance of abnormal capillary structures some days or weeks after irradiation *was* correlated well with the values of dose increment given above for skin reaction as determined split-dose experiments. Even more interestingly, these abnormalities had two peaks of intensity which occurred at about the same time as the two well-known peaks of skin desquamation, i.e. one at 4–5 weeks and a second peak at about 12 weeks. The first peak of vascular abnormalities therefore appears just *after* the first peak of skin reaction, whereas the second occurs just before or coincident with the second peak of gross skin reaction. This sequence is consistent with the hypothesis that the first peak of gross skin reaction is due primarily to depletion of the basal layer by direct killing of the cells by radiation, whereas the second peak of gross skin reaction might be due to vascular failure. This, however, is speculation. Ellis [24] and Van den Brenk [13] have pointed out the chicken-and-egg problem here; the whole connective tissue–vascular–basal epithelial layer complex appears to react together. If this is true, skin reactions are of more general relevance than to epidermal injury alone.

4. *Time–dose–number of fraction relationships*

The curve relating total dose to number of fractions obtained from pig skin reactions is similar to an average curve obtained by reviewing radiotherapy literature [12]. The curve of total dose versus number of fractions is not a simple straight line as Elkind's first-order theory suggests; nor a straight log–log line as Strandqvist [14] and Von Essen [25] suggested. Instead the curve was found both in the pig experiments and in the clinical data to decrease in slope (i.e. in dose increment) with greater numbers of fractions [12, 17, 26]. No simple equation with a single exponent would represent it precisely. Nevertheless, this curve of total dose versus number of fractions approximates fairly well to several of the empirical log dose versus log time slopes [24, 27] between 4 and about 30 fractions, and also to the iso-effect curve suggested by Elkind's cell repair theory. There is no great discrepancy between the estimates of several authors. Predictions about normal tissue tolerance doses can usefully be made, provided that the range of 4–30 fractions and 3–100 days is not exceeded.

5. *Time–dose–number relationships*

Let us now discuss the data which may be used as a guide to tolerance dose estimation in different schedules of fractionation.

A simple method is that of Fowler and Stern [12] where repopulation is taken into account to a limited extent. Table 3 shows the ratios of total doses as a function of the number of fractions, provided overall time does not vary

*Table 3. Multiplying factors for total dose*

| Number of fractions | Animal experiments and modal clinical data |
|---|---|
| 30 | 1·00 |
| 25 | 0·96 |
| 20 | 0·91 |
| 15 | 0·85 |
| 12 | 0·80 |
| 10 | 0·76 |
| 8 | 0·72 |
| 6 | 0·67 |
| 5 | 0·62 |
| 4 | 0·58 |
| 3 | 0·52 |
| 2 | 0·46 |
| 1 | 0·33 |

[see ref. 28].

by more than $\pm 50\%$. Fowler [28] discusses the limitations in detail. These values have worked well in practice, for example, in estimating the reduction in total dose on changing from 5 fractions per week to 3 fractions per week in the British Institute of Radiology Laryngo-Pharynx Fractionation Trial. It was estimated that to change from 20 to 12 fractions, for example, both schedules being given over 4 weeks, would require a reduction of about 12% in total dose. An iso-survival cell calculation, however, using $D_0 = 140$ rads and an extrapolation number of $N = 2 \cdot 8$, suggested a reduction of 20% instead. This order of difference can be distinguished radiotherap-

eutically, and the values from Table 3 suggesting 12% have been found to be reliable.

A more flexible method is that of Ellis [24], because it also takes overall time into account. The values are related by the formula:

$$\text{Total dose} = \text{N.S.D.} \times N^{0.24} \times T^{0.11}$$

where N.S.D.=a nominal single dose depending upon the organ and the patient,

$N$=number of dose fractions, and
$T$=overall time in days.

This is an empirical formula. Since time is a relatively small factor, as demonstrated above in the pig skin experiments, any uncertainty in the 0·11 exponent (i.e. in the proliferation rate) has little effect. It should be emphasized that because this exponent is small its accuracy is not important. For example, if overall time $T$ is doubled or halved, $T^{0.11}$ only increases or decreases by 8%.

Experiments on mouse skin to investigate the rate of repopulation of the basal layer cells in mouse epidermis have been carried out. The results of split dose experiments, where the interval was varied from a few hours to 28 days, are shown in Fig. 5 [18]. The "iso-effect" lines are drawn on a log dose v. log time scale to allow a comparison with Ellis' suggestions of the value of 0·11 for the exponent for normal tissues. In mouse skin, after a first dose of 1000 rads, there was reasonable agreement with this value (Fig. 5). In pig skin, the exponent had been found to be in the range of 0·09–0·18. Emery and co-workers [19] at Hammersmith have used the skin clone counting technique devised by Withers [20], and results of their split-dose experiments on mouse skin are also not inconsistent with an exponent of 0·11.

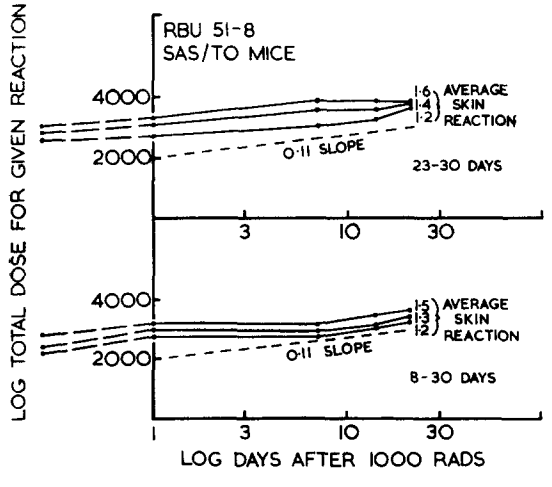


*Fig. 5. Two-dose experiments on mouse skin desquamation. Total dose for a given skin reaction over 23–30 days (upper) or 8–30 days (lower) is plotted against days after 1000 rads first dose on a log scale. Three levels of average skin reaction are shown. Single-dose values have been plotted at 0·3 day* [18].

In using such conditions, it should be remembered that most of the data above refer to the fairly rapidly renewing cell population of the basal layer of the epithelium. The tolerance–time–number relationships given above will not be quite correct for *very slowly* repopulating tissues, although the effect of proliferation has been shown to be rather small in determining the X-ray doses which normal tissues will tolerate. The caution should be added that at very low dose rates (a few rads per hour) it is possible that less early reaction would appear in a renewing population such as skin than is seen at the dose rates of 10–200 rad/min normally used; yet significant damage would accumulate in structures having less or no capability of cell repopulation. Early skin reactions might therefore not be a good guide to tolerance doses where very low dose rates or very extended fractionation regimes were used. There is no evidence that this caution refers to fractionation schedules so far used in radiotherapy. It may be concluded that reasonable predictions can be made of the total doses required to produce severe but tolerable reactions in many normal tissues, using fractionation schedules consisting of equal dose fractions given at approximately equal intervals.

## 3. TUMOURS

The use of different time–dose relationships would lead to no advantage in radiotherapy unless the injury to the tumour could thus be enhanced relative to that in normal tissues. It is therefore necessary to know about changes of tumour response as a result of alterations in fractionation, and to compare such changes with those described above in normal tissues.

Cell proliferation must of course be taken into account, as in the case of normal tissues. The kinetic parameters in tumours will in general be different from those relating to normal tissues, although similarities might occur between normal tissues stimulated to proliferate by radiation injury and some tumour tissues. Variations in vascularization in different parts of a tumour and at different times would be expected to play a major role both in influencing proliferation rates by nutritional limitation, and in determining radiosensitivity of cells by the degree of oxygenation.

### 1. *Cell population kinetics in tumours*

Denekamp [11] has investigated in our laboratories the cell population kinetics of four

*Table* 4

| Denekamp (1968) | | $RIB_5$ | SSO | $SSB_1$ | | $C_3H$ |
|---|---|---|---|---|---|---|
| Volume doubling time | $T_d$ | 24hr | 34hr | 78hr | | 108hr |
| | | | | rim | core | |
| Labelling index | *LI* | 30% | 16% | 16 | 5 | 16% |
| Cell cycle time | $T_c$ | 13·6 hr | 20·5 hr | 39 | 39 | 16·5 hr |
| Synthesis time | $T_s$ | 8·7 hr | 7·5 hr | 10 | 10 | 6·8 hr |
| Potential doubling time | $T$ | 24 hr | 36 hr | 48 | 152<br>58 hr | 33 hr |
| Growth fraction | *GF* | 46% | 48% | 76% | 20%<br>60% | 37% |
| "Loss factor"<br>$\varphi = 1 - T/Td$ | | 0 | −0·06 | 0·25 | | 0·7 |

rodent tumours having a series of volume doubling times (1, 1·5, 3·3 and 5 days respectively), in an attempt to correlate their response to irradiation with histological and kinetic parameters. Her results for unirradiated tumours are shown in Table 4 [11]. Results for irradiated tumours were similar when the tumours had regrown to the size at which they were irradiated.

The tumour described in the third column of Table IV, the rat fibrosarcoma $SSB_1$, had a proliferating rind and a relatively acellular core, and two columns of kinetic parameters are therefore given. Its relatively slow growth was largely due to the presence of the core which contained relatively few proliferating cells; after 1500 rads of X-rays it did not shrink at all. The tumour listed in the fourth column of Table 4 is a mouse mammary carcinoma, and is the most interesting of the four in the present context. It shows a large "discrepancy factor" (called "cell loss factor" by Steel [29]). The observation is that the rate of increase of tumour volume predicted from the birth rate of cells estimated using the values of labelling index, $T_s$ and $T_c$, differs appreciably from the observed rate of volume growth. A volume of material equivalent to 70% of the volume of cells produced by cell division is subsequently removed from the tumour volume, by unspecified processes, within the volume doubling time. It is not clear how this material is removed from the tumour. The relatively rapid removal of material is, however, consistent with the observation that the tumour shrinks rapidly (starting within one day) after a single dose of 1500 rads. The interesting correlation is with the fast rate of shrinkage and large extent of re-oxygenation in this tumour, as described below.

2. *Hypoxic cells in tumours*

It has been shown that hypoxic cells are important in determining the effects of large single doses [30–32]. Whether they are important in determining the effects on tumours of multi-fraction treatments is now being investigated intensively in several institutes.

Figures 6 and 7 show simple calculations relating to the idealized effects of repopulation and hypoxic cells in tumours. A mixed cell population containing 99% oxygenated and 1% hypoxic cells is assumed to be given 6 × 600

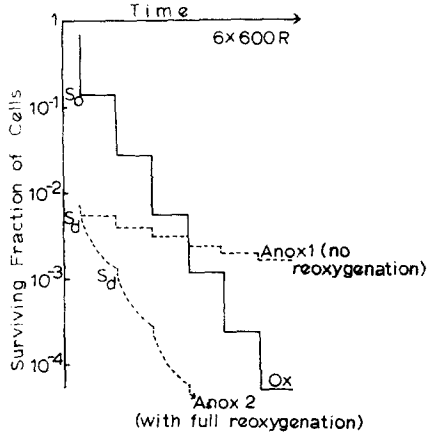


*Fig.* 6. *Results of computer programme shown graphically. Each dose of* 600 *R reduces the surviving fraction of oxygenated cells to* $S_o$, *and of anoxic cells to Sd. No repopulation is assumed here. If none of the anoxic cells become oxygenated as the treatment proceeds, the curve "Anox* 1" *is obtained. If, however some anoxic cells become oxygenated between successive fractions, so that the proportion of anoxic cells is kept about* $10^{-2}$ *of the surviving aerobic cells, then the curve "Anox* 2" *is obtained, and the anoxic cells* never *become predominant. Practical cases will lie between these extremes* [33].

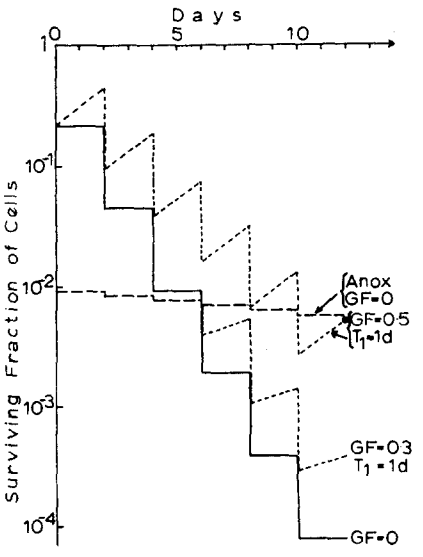


*Fig. 7. As in Fig. 6, but assuming a wide range of growth fractions (G.F.=0, 0·3 and 0·5) for the oxygenated cells.*

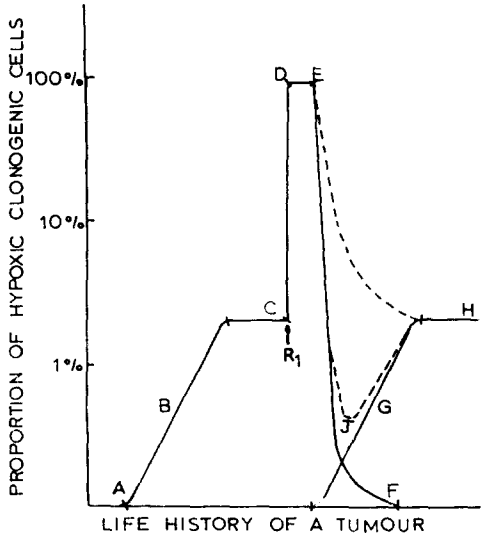


*Fig. 8. Diagrammatic representation of changes in the proportion of hypoxic clonogenic cells during the life history of a solid tumour irradiated with a large single dose of X-rays at a time denoted by $R_1$. The minimum level to which the hypoxic proportion falls after treatment is probably determined by the type of tumour and the size of dose used and could possibly be lower (J) than the level (C) existing just before irradiation (based on Thomlinson [40]).*

rads. The degree of hypoxia is assumed to be sufficiently low to give the fully protective increase of a factor of 2·5 in dose required to kill a given proportion of cells, yet not such that cells protected from radiation death cannot survive in the post-irradiation environment. The calculated change in the number of surviving cells with time is shown in Fig. 6. In the curve "anox 1" the hypoxic cells were assumed to remain hypoxic throughout the six fractions, and they soon predominate. The curve "anox 2" shows the decrease in their proportion with time if the tumour is assumed to become re-oxygenated after each dose, so as to restore the hypoxic proportion to 1% when each of the dose fractions is given. In this case, the effect of well-oxygenated cells predominates. Thus the effect of hypoxic cells is either decisive or negligible, depending completely on *how effective is the re-oxygenation of hypoxic cells.*

Figure 7 shows the effect of assuming various growth fractions where G.F. is defined as the proportion of oxygenated cells which is proliferating. The cell cycle times are assumed to be all equal to the fraction interval, for simplicity. Here the largest difference in surviving cell number due to differences in G.F. is by a factor of about $10^2$, as compared with $10^4$ for the effects of different degrees of re-oxygenation of hypoxic cells shown in Fig. 6. As was shown above for normal tissues, in all except very rapidly proliferating tumours the proliferation rate has a smaller effect than the intracellular repair after each fraction, and we may now conclude that it has in general less effect than that of re-oxygenation. This suggests that the change in oxygen status of cells in a tumour with time after irradiation is one of the most important factors to investigate.

Thomlinson [34] has proposed a hypothetical sequence of changes in oxygenation in a tumour with time after irradiation (Fig. 8). He has also proposed experiments for testing the hypothesis. Howes has followed his method in outline, using however a different biological end-point.

Howes [35, 36] has studied the reoxygenation in transplanted mouse mammary tumours which takes place after a single or a fractionated dose of 1500 rads of 250 kV X-rays. In these studies the tumours, which were first-generation transplants having a mean volume doubling time of 5 days, were irradiated with the priming dose when their diameter reached $6\pm1$ mm. After various intervals of time each tumour was treated with one of a series of test doses given under conditions either of hypoxia produced by clamping, or air-breathing, or high pressure oxygen breathing at three atmospheres absolute pressure. The range of doses in each case was chosen to allow estimates to be made of the dose required to cure 50% of the animals. The tumours were tested by irradiating them at one of the three conditions of oxygenation and at a given time after the priming dose of 1500 rads. "Cure" was defined as absence of a palpable tumour mass at 150 days. Suit *et al.* [37] have discussed this end-point, the $TCD_{50}$/150 days. The ratio of $TCD_{50}$ Air to the other values in

the series $TCD_{50}$ Hypoxia: $TCD_{50}$ Air: $TCD_{50}$ $HPO_2$ gave an indication of the relative proportion of hypoxic cells at the time of testing. If a simple cell survival model is assumed, with survival curves of slope 117 rads and 350 rads respectively for the oxygenated and the hypoxic components, estimates can be made of the "effective proportion" of hypoxic cells present and viable at the various times of testing.

Other interpretations of these results are possible; for example, it may instead be assumed that the distribution of oxygen among surviving cells is uniformly low instead of being divided into "hypoxic" and "oxygenated" compartments [35, 36]. For the present discussion, however, it has been assumed that two distinct populations of tumour cells exist, the cells in one population being protected from radiation injury by hypoxia.

The results after a single dose of 1500 rads (Fig. 9) shows that immediately after irradiation the proportion of hypoxic cells rises to about 100%, as expected. A few hours later, however, a significant number of the surviving hypoxic cells have become re-oxygenated. By 48 hr the proportion of hypoxic cells is significantly lower than that existing in the unirradiated tumours; this situation gives rise to a marked increase in radiosensitivity in comparison with the unirradiated tumours. The overall pattern of re-oxygenation observed is in accordance with that predicted by Thomlinson (Fig. 8).

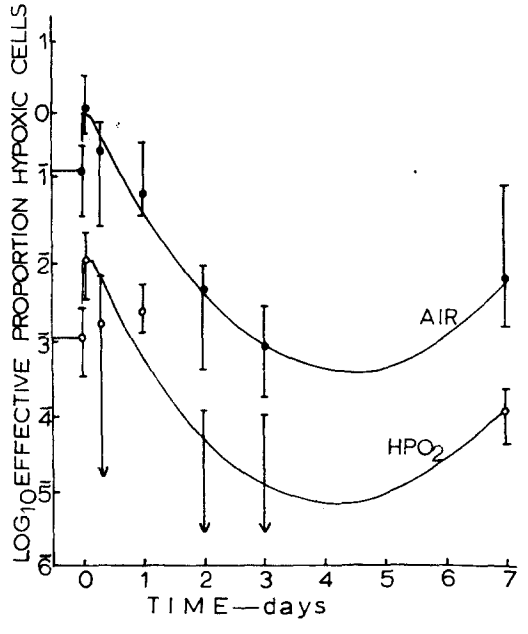


*Fig. 9. Changes in the estimated proportion of hypoxic cells in transplanted $C_3H$ mouse mammary tumours given a priming dose of 1500 rads. The values have been obtained by using a theoretical cell survival model with parameters $D_0$ (hypoxic) = 350 rads, oxygen enhancement ratio (m) = 3·0. The vertical bars indicate 95% confidence limits estimated from the corresponding error in $TCD_{50}$ values. The upper curve (full circles) shows results obtained with air breathing conditions, the lower curve (open circles) with high pressure oxygen.*

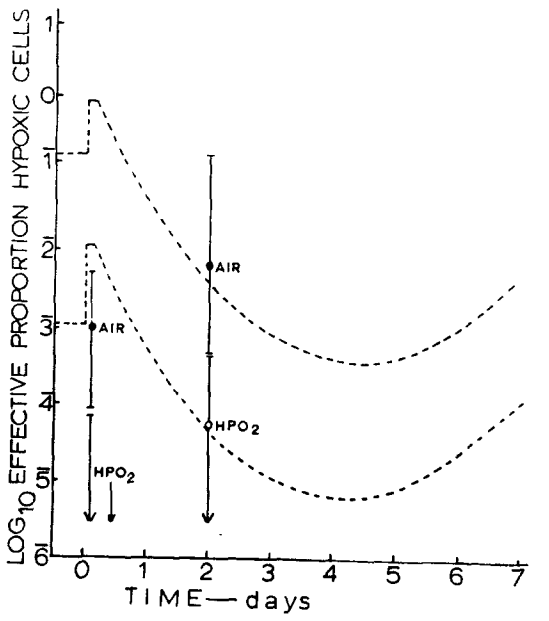


*Fig. 10. Values of the estimated proportion of hypoxic cells at 30 min and 48 hr after treatment with 5 × 300 rads in 9 days. The dashed line refers to results obtained after 1500 rads given as a single exposure (Fig. 9).*

Figure 10 shows the estimated proportion of hypoxic cells at various times after the last of 5 × 300 rads given at 48 hr intervals. It was found that at ½hr after the last dose (the shortest practicable interval) the proportion was significantly lower than at the beginning of treatment. These results imply that extensive re-oxygenation took place during the fractionated priming treatment. By 7 days after the last of the 5 fractions the proportion of hypoxic cells had returned to approximately its original value lower (not shown in Fig. 10).

The probability of cure is determined by the *total numbers* of hypoxic cells rather than by the proportions. Estimates of total numbers of hypoxic cells can be made using a similar cell survival model as for the estimation of hypoxic proportions. Figure 11 shows the calculated cell numbers at different times after the single priming dose of 1500 rads. Since the total number of cells in the tumour will normally increase with time, due to repopulation, the graphs of cell numbers will be higher at later times than the graphs of proportions shown in Figs. 9 and 10. The time sequence of total numbers differs from that for hypoxic proportions in Fig. 9 in that the minimum value (which corresponds to the time at which the tumour is most radiosensitive) is now at about two days instead of beyond three.

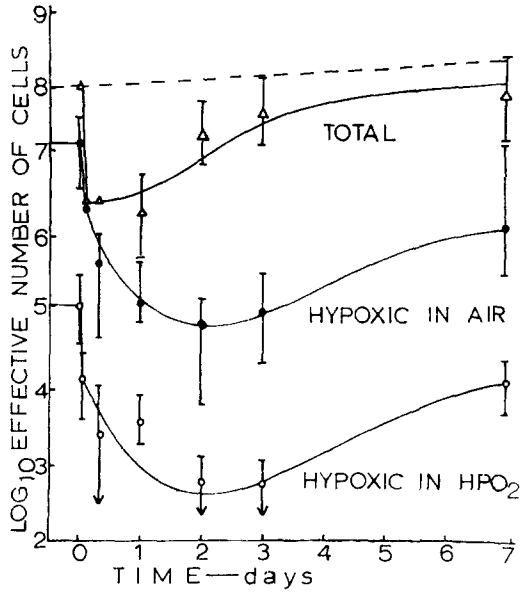


*Fig.* 11. *Estimated changes in the total numbers of viable cells in transplanted $C_3H$ mouse mammary tumours after irradiation of a single dose of* 1500 *rads of X-rays. The upper dashed line represents the growth of untreated tumours (volume doubling time* 6 *days). The open triangles indicate values of the total number of cells existing in the tumours assuming the initial population contained* $10_8$ *viable tumour cells. The full circles indicate values of the effective total number of hypoxic cells with air breathing, and the open circles indicate values during high pressure oxygen breathing.*

The total numbers of cells estimated after the 5×300 rad fractionated regimes (Fig. 12) indicates that the time at which these tumours were most sensitive was probably earlier than 2 days after the last treatment. It should be noted that the total number of cells surviving

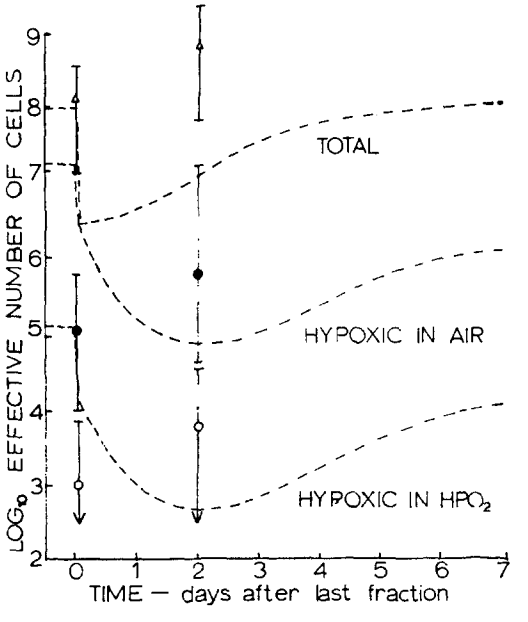


*Fig.* 12. *Estimated values of the total numbers of tumour cells at* 30 *minutes and* 48 *hr after irradiation of transplanted $C_3H$ mouse mammary tumours. The dashed lines represent results obtained with* 1500 *rads given as a single exposure.*

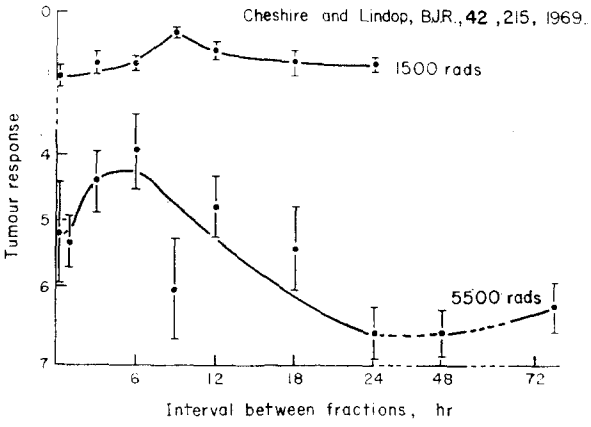


*Fig.* 13. *Response of spontaneous $C_3H$ mouse mammary tumours measured in terms of regrowth after X-irradiation with single or divided doses. All irradiations were performed with anaesthetized mice breathing pure oxygen at normal atmospheric pressure.*

after the fractionated schedule was greater than after the single dose of 1500 rads due, presumably, to the effects both of intracellular repair after each fraction and of repopulation during the 9 days of treatment.

It appears that in this type of carcinoma the hypoxic tumour cells can be very effectively re-oxygenated. Other studies of the response of transplanted and spontaneous $C_3H$ mouse-mammary tumours to two doses of radiation separated by time intervals of 0 to 72 hr (Fig. 13; i.e. Fig. 4 in [38]) are in agreement with this conclusion.

Figure 14 shows the results of Suit and Schiavone [39] who experimented with mouse-mammary tumours transplanted into the thigh muscle. They found a slight improvement in oxygenation for air-breathing mice tested at

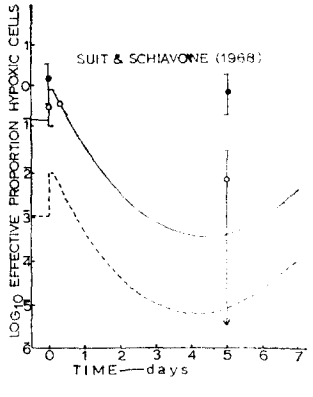


*Fig.* 14. *Estimates of the proportion of hypoxic cells in transplanted $C_3H$ mouse mammary tumours obtained using data published by Suit and Schiavone* [39]. *The tumours were given either single doses of X-rays, or were treated with a priming dose of* 3000 *rads under high pressure oxygen breathing conditions followed with a range of test doses after an interval of* 5 *days. Full circles denote results for air-breathing mice, open circles for mice breathing high pressure oxygen. The point at 6 hr is similarly taken from unpublished data kindly supplied by Drs. Suit and Urano.*

5 days after a priming dose of 3000 rads given under $HPO_2$ conditions. (A more marked improvement was found when the test doses also were given under $HPO_2$ conditions.) Their results showed a less complete oxygenation at 5 days than in our mouse-mammary tumours which were given 1500 rads as a priming dose, when the test doses were given while the mice were breathing air. There were important differences between the two tumour systems however. Suit and Schiavone transplanted into muscle instead of into subcutaneous tissue; they did not obtain cures after single doses even with 3000 rads $HPO_2$; and they found no decrease in tumour volume after this dose.

Studies with other types of rodent tumour have yielded variable results. Thomlinson [40] working with the rapidly growing transplantable rat fibrosarcoma $RIB_5$ (first column of Table 4) has observed an increase in hypoxic proportion at 40 minutes after a single dose of 1500 rads followed by a fall to approximately the original value in 24 hr, in qualitative agreement with his original model (Fig. 8). As yet there is no evidence of the hypoxic proportion falling below its original value in this tumour.

Another rapidly growing transplantable fibrosarcoma growing in $C_3H$ mice, has been studied by Kallman and Bleehan [10] and Van Putten and Kallman [41]. The hypoxic proportion before and after both single and fractionated doses of X-rays has been estimated using the tumour cell dilution technique developed by Hewitt and Wilson [42]. Changes in the hypoxic proportion after 1000 rads single dose are shown in Fig. 15. These data are preliminary but more recent evidence (Kallman, personal communication) confirm the pattern described. It can be seen that re-oxygenation is rapid, the original level being reached by approximately 6 hr; but re-oxygenation did not proceed beyond the pre-irradiation level in the time studied. These tumours continue to grow larger for 2 or 3 days after the single dose. This post-irradiation swelling is also observed in the rat fibrosarcoma $RIB_5$, but not in the $C_3H$ tumours used in the present work (Figs. 9–12).

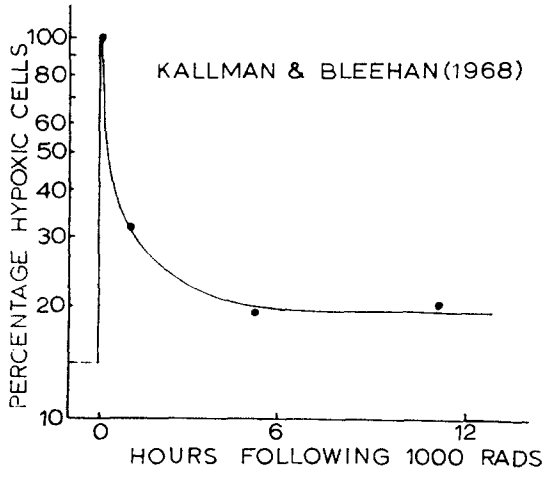


*Fig. 15. Changes in the proportion of hypoxic cells in a transplanted mouse fibrosarcoma after irradiation with a single dose of* 1000 *rads (from Kallman and Bleehan* [10]).

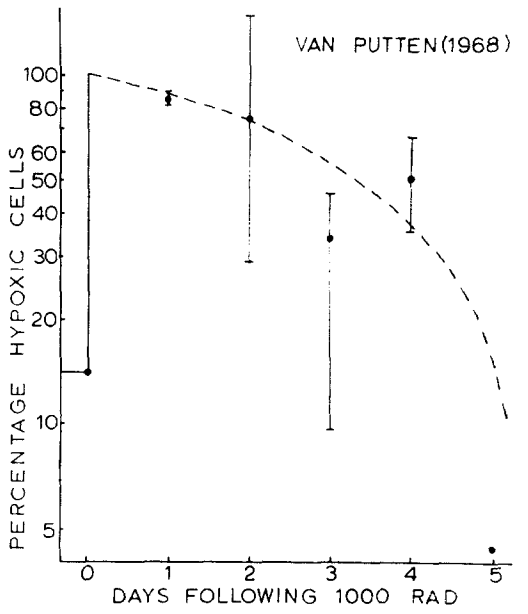


*Fig. 16. Changes in the proportion of hypoxic cells in a transplanted mouse osteosarcoma after irradiation with a single dose of* 1000 *rads. The vertical bars indicate the range of values observed at each time interval (from Van Putten* [44]).

Van Putten [42, 43] using similar techniques has found a different time sequence for the estimated proportion of hypoxic cells in a transplantable mouse osteosarcoma after treatment with a single dose of 1000 rads (Fig. 16). No significant re-oxygenation takes place before 2 days after irradiation. This tumour, therefore, forms an exception to other observations of rapid re-oxygenation.

### 3. *Extension to multi-fraction schedules*

No detailed study has yet been made of changes in oxygenation taking place during a multi-fraction course of treatment. However, sufficient knowledge is available from some rodent tumours to allow preliminary assumptions to be made. Thus, in order to account for the low value of the hypoxic proportion at the end of the $5 \times 300$ rad fractionated treatment of the transplanted $C_3H$-mouse mammary tumours mentioned above (Fig. 10), it is assumed that the hypoxic proportion rises immediately after each dose and then falls to a

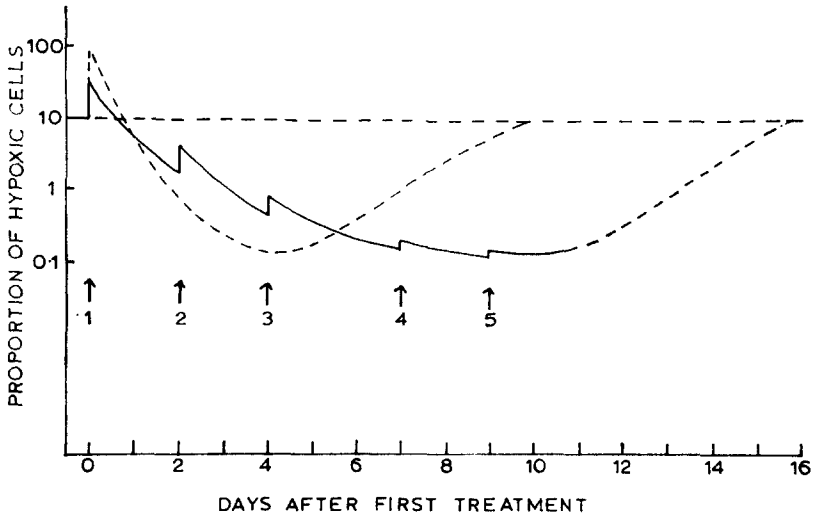


*Fig. 17. A possible scheme of re-oxygenation taking place during 5 × 300 rad priming treatment given to transplanted C3H mouse mammary tumours. For comparison, the pattern obtained after a single dose of 1500 rads is also shown (dotted line) on the same scale.*

level which is either equal to, or lower than, that existing immediately before each treatment (Fig. 17). In this figure the vertical increments indicate the hypoxic proportion present at the time of each dose. They become successively smaller since it is assumed that the proportion of hypoxic cells is progressively reduced throughout the regime.

Field *et al.* [22] have investigated the volume response of rat fibrosarcoma $RIB_5$ treated either with single or with 5 daily doses of X-rays or fast neutrons. The results were interpreted, in the light of the pattern of re-oxygenation after single doses, as being due to a rise in the hypoxic proportion after each treatment with a fall within 24 hr to a level which was still higher than that appertaining before the treatment (Fig. 18). In Fig. 18 it is assumed for simplicity that equal proportions of hypoxic cells are present at the time of each dose fraction; it is unlikely of course that this equality is real.

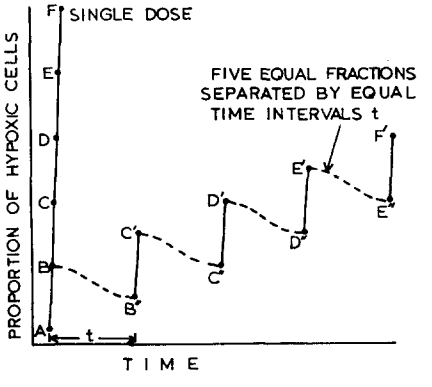


*Fig. 18. Schematic diagram of the alteration in proportion of hypoxic cells in the transplanted rat fibrosarcoma, $RIB_5$, during a single dose as compared with the alterations after each of five equal fractions, assuming the five alterations are equal to each other (reproduced from Field* et al. [22]).

The response of the C3H mouse-mammary tumours to fast neutrons has not been tested, and the changes in hypoxic proportion with time after single X-ray doses of 300 rads are still under investigation. Nevertheless, it appears that the 5 × 300 rad fractionated X-ray regime is relatively effective in this tumour. This is probably due to the enhanced sensitivity found in this tumour due to the fact that the hypoxic proportion can fall below the initial proportion of hypoxic cells in unirradiated tumours. The large degree of re-oxygenation might, in turn, be associated with the rapid shrinking of this tumour after a single dose.

It is now clearly established that re-oxygenation is an important, indeed predominant, factor in determining the response of at least some experimental rodent tumours to fractionated irradiation. A similar conclusion concerning the radiotherapy of human tumours cannot, of course, be drawn. However, it is reasonable to suggest that the improved therapeutic response obtained with fractionated regimes over single dose treatments might be due, at least in part, to an increase in tumour radiosensitivity accompanying re-oxygenation.

That such an assumption is not inconsistent with clinical results is illustrated by an analysis of the dose vs. time relationship for recurrent nodules of human mammary carcinoma [45]. In Fig. 19 an iso-effect line redrawn by Howes and Field [46] from Friedman and Pearlman's clinical data is compared with iso-effect lines for the response of animal tumours and for both animal and human skin. The point is that the tumour isodoses might be lower than those

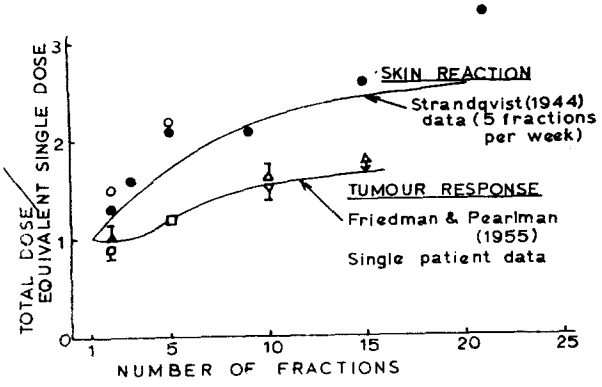


*Fig. 19. Dose–time relationships for the response of skin and experimental tumours compared with a similar relationship for treatment of human mammary carcinoma obtained by Friedman and Pearlman (reproduced from Howes and Field* [46]).

for normal tissues due to greater sensitization by re-oxygenation for fractionated schedules than for single doses whilst prompt repair might be similar for both types of tissue. The close agreement of the animal and clinical data encourages the hypothesis that the processes taking place during fractionated treatment might be similar for both systems, and that general relationships for certain types of tumour might be possible to discover.

Van den Brenk [13] has pointed out that small primary human tumours are often easier to eradicate than would be expected if hypoxic cells played an important part in their response to radiation. He emphasized the difference in this respect between small human tumours and the experimental rodent tumours of comparable absolute size. We wish to point out a further difference which we believe to be relevant: the experimental rodent tumours are much more rapidly growing than most human tumours.

If it is indeed found that re-oxygenation is a major factor in human tumour response, then a precise knowledge of its properties will be necessary in order to form a rational basis for optimal schemes of fractionation. It is not possible to make definite suggestions at present, but if intervals of 2 to 3 days are advantageous in mouse tumours with a doubling time of 5 days, then it seems reasonable to consider intervals longer than 1 day for treating the slower-growing human tumours.

It is however a matter of great concern that no really satisfactory experimental models representing slow-growing human tumours are at present available.

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## SUMMARY

*At present the doses prescribed by radiotherapists are determined by the maximum tolerable reactions of normal tissue rather than by the response of the tumour. Combinations of total dose (in several fractions) and overall times required for normal tissue tolerance in various fractionation schedules can now be predicted with fair reliability. The radiobiological basis of such relationships are reviewed. The magnitudes of cellular repopulation and of prompt repair from intracellular radiation injury are compared. It is shown that the latter effect is the greater for conventional fractionation regimes, and also for regimes with smaller numbers of larger dose fractions.*

*The response of a tumour will vary with the size of the dose and the length of the interval between doses according to the dynamics of the cell population and changes in its oxygenation. In order to achieve better therapeutic ratios radiotherapists will need to take into account these properties of tumours together with the somewhat better established time–dose relationships for normal tissues.*

*Some improvement in oxygenation of tumours has been demonstrated after irradiation. The possible effects of this re-oxygenation upon optimum fractionation are discussed, with special reference to recent experimental results from transplanted mammary tumours in $C_3H$ mice.*

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# Further Cytogenetical Investigations in Polycythaemia Vera

SYLVIA D. LAWLER, ROSEMARY E. MILLARD and H. E. M. KAY

*Department of Clinical Research, The Royal Marsden Hospital and The Institute of Cancer Research, London S.W.3, England*

## INTRODUCTION

IN PREVIOUS studies [1, 2] we have demonstrated the presence of chromosomal abnormalities in the bone marrow cells of both treated and untreated patients with polycythaemia vera (P.V.). Apart from sporadic cells showing radiation damage in patients treated with $^{32}P$, clones of cells showing the same abnormality have been demonstrated in several patients. In untreated cases these clones are composed of aneuploid cells, the patients having normal chromosomal complements in other tissues. In irradiated patients the clones are characterised by structurally altered chromosomes, the abnormality presumably having been induced by the treatment. An abnormality involving a deletion of one of the chromosomes of the F group, (F?—), has been observed in several treated patients and thus appears to have some degree of specificity for P.V., since it occurs but rarely in other conditions. The studies have been extended to a total number of 79 cases; serial observations of some of these have been made over a period of up to four years. It has thus been possible to observe the behaviour of chromosomally marked populations of cells in relation to the progress of the disease.

## RESULTS

Material and methods are as described previously [1], the bone marrow chromosome preparations being made by a direct method. In 33 cases the chromosomes were first examined before treatment; 46 patients had already been treated, all but one with $^{32}P$, and a a few patients had in addition received busulphan or X-rays. Except in cases of leukaemia, all the results apply to *bone marrow preparations*. Phytohaemagglutinin was not used in the blood cultures recorded in the tables. Details of the chromosome studies are given only in certain cases with clonal abnormalities. The nomenclature used in describing the chromosome abnormalities follows the suggestions of the Chicago Conference [3]. Where case numbers are used they correspond to those in our previous publications [1, 2]. Some information has already been given for cases 1–43 [1] and also cases 46, 48 and 54 [2]: the remaining cases have not been described previously.

1. *Thirty-three untreated patients*

| | Normal chromosomes | Aneuploidy |
|---|---|---|
| Females | 12 | 4 |
| Males | 16 | 1 |

In the four females, the aneuploidy took the form of one or more extra C-group chromosomes, involving the entire cell population in two cases (No. 10 and No. 11) and mixed with normal cells in the other two (No. 3 and No. 9). In the male (No. 8) with aneuploidy, all the cells lacked a Y chromosome in the first bone marrow aspirate.

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*Table* 1. *Chromosomal analysis of serial B.M. samples in a patient presenting with aneuploidy (case* 3)

| Date | Hb g/100 ml | WBC (mm³) | Plats. (mm³) | Chromosomes of analysed cells <46* | 46 | >46 | Total |
|---|---|---|---|---|---|---|---|
| August 1965 | 19·1 | 55,000 | 353,000 | 1. 44<br>2. 45 | 30. normal. | 1. 47, XX, C+<br>5. 48, XX, C+2 | 39 |
| November 1965 | 17·8 | 42,000 | 1,483,000 | 1. 41<br>1. 44<br>9. 45 | 29. normal | 1. 47, XX, C+<br>1. 48, XX, C+2 | 42 |
| March 1968 | 17·5 | 22,700 | 322,000 | 1. 44, F?−<br>2. 45<br>2. 45, F?− | 13. normal<br>33. XX, F?−<br>1. XX, 3+, B+, G−2 | 1. 48, XX, B+2<br>7. 48, XX, C+2<br>1. 48, XX, C+2, E+, D−<br>10. 48, XX, B+, C+<br>1. 49, XX, C+2, Mar1+ | 72 |
| July 1969 | 16·4 | 7800 | 273,000 | 1. 45 | 10. normal<br>11. XX, F?− | 1. 48, XX, B+, C+<br>1. 48, XX, C+, Mar1+<br>3. 49, XX, C+2, Mar1+ | 27 |

*The analyses of these cells are not given. The chromosome losses were random.

*Follow-up studies of the patients who showed aneuploidy when untreated*

*Case No.* 3. Details of serial studies on this patient are given in Table 1. She was treated with $^{32}P$ between the time of the first and second aspirate. At the time of the second aspirate, November 1965, the proportion of hyperdiploid cells had decreased. In the third aspirate, March 1968, the F?− abnormality was detected for the first time and was found only in diploid or hypodiploid cells. The proportion of hyperdiploid cells had increased and a new karyotypic variant 48, XX, B+, C+, illustrated in Fig. 1, was found. A marker chromosome, of C-group size with a subterminal centromere, was observed in one hyperdiploid cell. Sixteen months later, July 1969, this marker chromosome was present in four out of five hyperdiploid cells found amongst the twenty-seven cells analysed.

In the first bone marrow aspirate seven polyploid cells were observed, six of these were regular 4n–16n cells, but one cell with 96 chromosomes had four more C-group chromosomes than would be expected in a regular tetraploid cell. In the third aspirate, March 1968, all the polyploid cells had one or more F?− chromosomes and were of regular ploidy.

*Case No.* 8. The bone marrow of this constitutionally normal male patient, initially entirely populated by 45,X,Y— cells, was aspirated on five different occasions. In some of the aspirates the population was a mixture of normal male cells and 45,X,Y— cells. The proportion of 45,X,Y— cells in relation to the haematological findings is shown in Table 2. It will be noticed that the proportion of 45, X,Y— cells varied and that the more normal haematological picture was associated with decrease in frequency of these abnormal cells. Polyploid cells with and without Y chromosomes were detected in the later samples, but in the pre-treatment sample all the polyploid cells lacked Y chromosomes.

2. *Forty-six patients first examined after treatment had commenced*

The chromosomal findings in these patients may be classified into five categories:

(a) Normal chromosomes — 14 cases.

(b) Predominantly normal cells but with occasional cells showing radiation damage — 16 cases.

(c) Clones of cells with F?− abnormality — 7 cases.

(d) Other major clonal abnormalities — 5 cases.

(e) Gross chromosomal abnormality associated with leukaemia — 4 cases.

*Follow-up studies of patients with the F?− abnormality*

During the course of the study the F?− abnormality has been observed in 10 patients, viz: the 7 treated patients in whom the abnormality was detected in the first bone

*Table 2. Serial studies in a male patient with 45,X,Y — bone marrow cells (case 8)*

| Date | Total $^{32}P$ | Last dose $^{32}P$ and interval | Hb. (g/100 ml) | WBC ($mm^3$) | Plats. ($mm^3$) | No. of cells analysed | %45,X,Y— cells | Polyploid without Y | Polyploid with Y |
|---|---|---|---|---|---|---|---|---|---|
| February 1965 | — | — | 23·2 | 15,800 | 1,400,000 | 15 | 100 | 6 | — |
| July 1965 | 9 mCi | 4 mCi 4 mths. | 14·8 | 10,000 | 320,000 | 42 | 69 | — | — |
| January 1966 | 9 mCi | 4 mCi 10 mths. | 16·7 | 10,000 | — | 50 | 72 | 3 | 1 |
| August 1967 | 14 mCi | 5 mCi 8 mths. | 17·5 | 8200 | 438,000 | 25 | 100 | — | — |
| May 1968 | 18·5 mCi + venesection 4 pints | 4·5 mCi 3 mths. | 13·2 | 6600 | 268,000 | 50 | 66 | 7 | 4 |

*Table 3. Serial analyses of bone marrow cells of cases with F?— abnormality*

| Case No. | Date | Total $^{32}P$ and other treatment | Last dose $^{32}P$ | Interval | No. of cells analysed | % cells F?— |
|---|---|---|---|---|---|---|
| 3 | August 1965 | — | — | — | 39 (6*) | 0 |
| | November 1965 | 5 mCi | 5 mCi | 3 mths. | 42 (2*) | 0 |
| | March 1968 | 12 mCi | 7 mCi | 1 mth. | 72 (21*) | 70 (diploid cells) |
| | July 1969 | 19 mCi+busulphan 4mg daily Feb–April 1969 | 7 mCi | 16 mths. | 27 (5*) | 50 (diploid cells) |
| 12 | November 1964 | 12 mCi | 4 mCi | 2 yr | 42 | 0 |
| | August 1967 | 16 mCi | 4 mCi | 2 yr | 32 | 97 |
| 37 | April 1965 | 24·5 mCi | 3 mCi | 19 mths. | 52 | 65 |
| | May 1966 | 34·5 mCi | 5 mCi | ½ mth. | 53 | 89 |
| | November 1967 | 44·5 mCi | 5 mCi | 6 mths. | 50 | 100 |
| | July 1968 | 51·5 mCi | 7 mCi | 4 mths. | 49 | 98 |
| 39 | April 1965 | 47·5 mCi | 5 mCi | 2 mths. | 30 | 100 |
| | May 1966 | 47·5 mCi | 5 mCi | 15 mths. | 16 | 100 |
| 44 | November 1965 | — | — | — | 29 | 0 |
| | October 1968 | X-rays to spleen Dec. 1965 Busulphan 4mg daily 1966 & April–June 1968 | — | — | 60 | 17 |
| 46 | February 1966 | 24·5 mCi+phenylhydrazine | 3 mCi | 18 mths. | 64 | 89 |
| | November 1966 | 24·5 mCi | 3 mCi | 27 mths. | 22 | 100 |
| 48 | February 1966 | 30 mCi+84mg busulphan from Nov. 1965 | 5 mCi | 14 mths. | 50 | 22 |
| | January 1968 | 35·5 mCi+425 mg busulphan | 5·5 mCi | 2 yr | 58 | 82 |
| | March 1968 | 35·5 mCi+440 mg busulphan+100 mg B6 daily for 2 mths. | 5·5 mCi | 2 yr | 50 | 82 |
| 54 | July 1966 | Busulphan 4 mg daily April–July 1966 | — | — | 9 | 100 |
| | February 1968 | — | — | — | 38 | 50 |

*Cells with one or more extra B- or C-group chromosomes. (See Table 1).

*Table 4. Treated cases with definite evidence of abnormal diploid clone on first examination*

| Case No. | Age and sex | Length of history | Total $^{32}P$ and other treatment | Last dose $^{32}P$ and interval | Abnormality |
|---|---|---|---|---|---|
| 33 | 51 M | 15 yr | 64 mCi +busulphan | 10 mCi 7 yr | 8/11 cells 46, XY, Dq− |
| 34 | 66 M | 8 yr | 33 mCi | 5 mCi 17 mths. | 34/40 cells 46, XY, Cq− |
| 35 | 52 F | 17 yr | 16 mCi +XRT | 5 mCi 16 mths. | 10/29 cells 46, XX, Dq− |
| 58 | 54 M | 7 yr | 40 mCi | 10 mCi 4 yr | 11/100 cells 46, XY, 1q+ |
| 61 | 66 F | 15 yr | 56 mCi | 5·5 mCi 5 mths. | 10/21 cells 46, XX, C+, 16−, 18−, F+. = t(16?+, 18q−) |

marrow aspirate and three patients whose F group chromosomes were normal when they were first included in the series. These are Case No. 3, the follow-up studies having already been given in Table 1, Case No. 44 from the untreated group and Case No. 12 from the treated group.

The F?− abnormality is illustrated in Fig. 2; serial observations were made in eight of the cases showing this abnormality. In Table 3 details of the treatment received in relation to the percentage of F?− cells are recorded. It may be noted that the frequency of abnormal cells tended to increase during the period of observation. Case No. 54 is exceptional in that she was not irradiated. In Case No. 12 there was an additional anomaly, an apparent deletion in one of the C-group chromosomes.

*Patients with other major abnormal clones*

The clonal abnormalities detected in the bone marrow of these five patients are described in Table 4 together with data on the length of history and treatment. In all these cases, excepting No. 61, successful blood lymphocyte cultures were obtained. These showed normal karyotypes.

The amount of material missing from the deleted D-group chromosome was greater in Case No. 33 than in Case No. 35. The deleted C-group chromosome in Case No. 34 morphologically resembled a number 16 chromosome. In these three cases there was no visible evidence of a reciprocal translocation. In Case No. 58, the clone was characterized by an anomalous number 1 chromosome (1q+), a karyotype is illustrated in Fig. 3, and the A-group chromosomes from five cells are shown in Fig. 4. The origin of the additional chromosomal material could not be determined. In the majority of the cells the remainder of the chromosomal complement was normal. Case No. 61 appeared to have a balanced reciprocal translocation, illustrated in Fig. 5. The chromosome constitution of the clone is 46, XX, C+, 16−, 18−, F+. = t(16?+, 18q−).

Follow-up studies were carried out in 4 of the 5 patients.

*Case No.* 34. In Fig. 6 are shown the frequency of the clone marked by the Cq− chromosome and the haemoglobin, white cell and platelet counts at the time of aspiration. In relapse when the blood levels were high the clonal cells predominated. When the blood levels fell in response to treatment the frequency of the clonal cells decreased.

*Case No.* 35. Data on this patient are presented in Table 5. As in Case No. 34, the proportion of clonal cells was increased when the haemoglobin was high.

*Case No.* 58. Details are given in Table 6. In the three samples the frequency of the major clone of cells with the 1q+ chromosome varied between 11% and 25%: these percentages were based on scoring 100–200 cells for the presence or absence of the abnormal chromosome. The fluctuation in the proportion of the clonal cells did not appear to be related to the blood levels. In the latest sample, one-third of the cells with the 1q+ chromosome also showed a ring chromosome. During the period of observation two other minor clones lacking the 1q+ chromosome were observed. The A-, B- and C-group chromosomes of a cell representing the 46, XY, 2+, C− minor clone are illustrated in Fig. 7. The formula of the cells of the other minor clone was 46, XY, Dq−. In each sample approximately one-quarter of the polyploid metaphases showed one or more 1q+ chromosomes. Three polyploids in the latest sample had 1q+ chromosomes and also ring chromosomes.

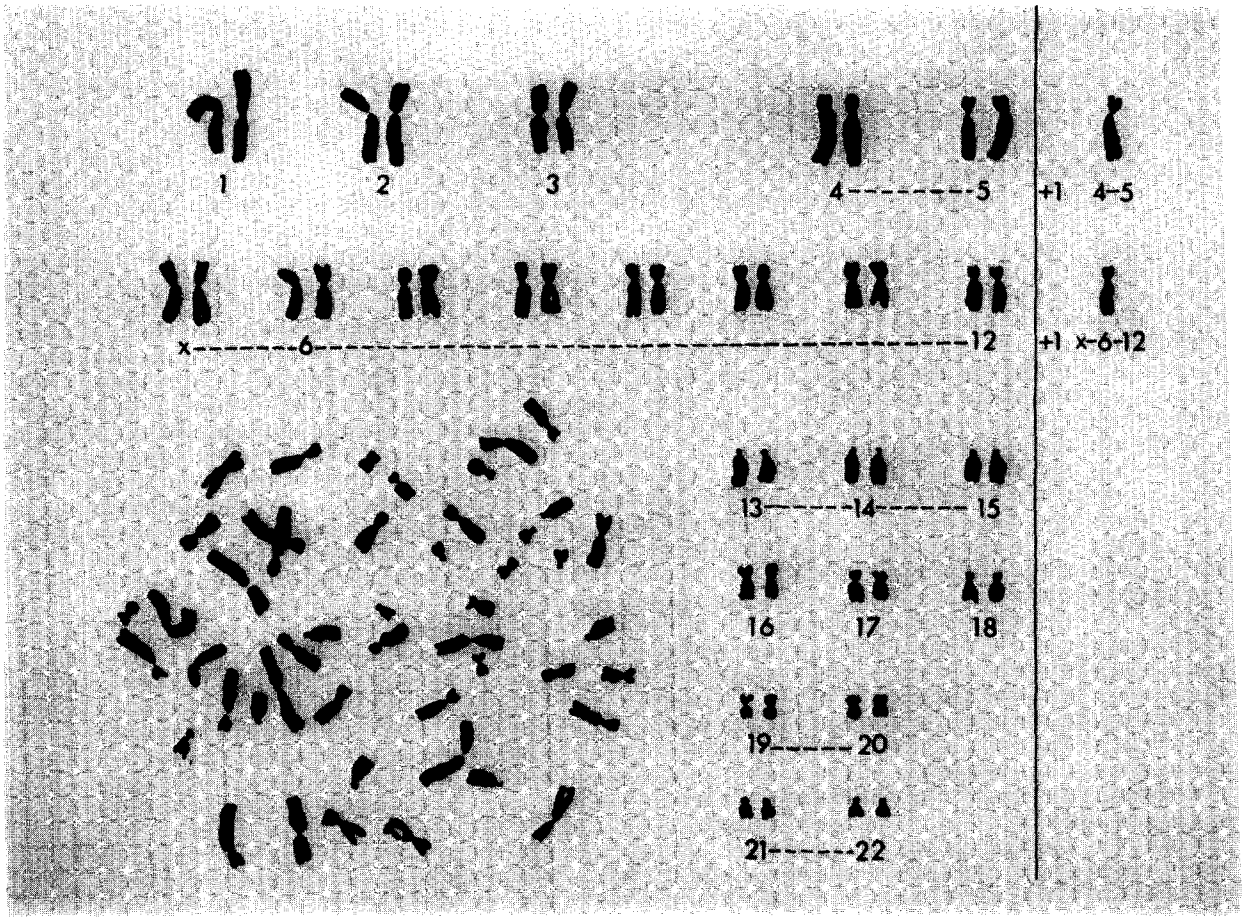


*Fig.* 1. *Karotype of cell with* 48, *XX*, *B*+, *C*+ *chromosome constitution from Case No.* 3.

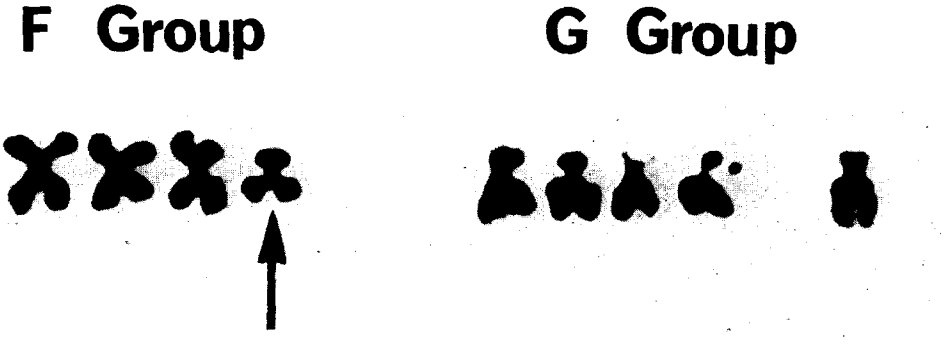


*Fig.* 2. *The F?— abnormality in Case No.* 48.

(to face p. 226)

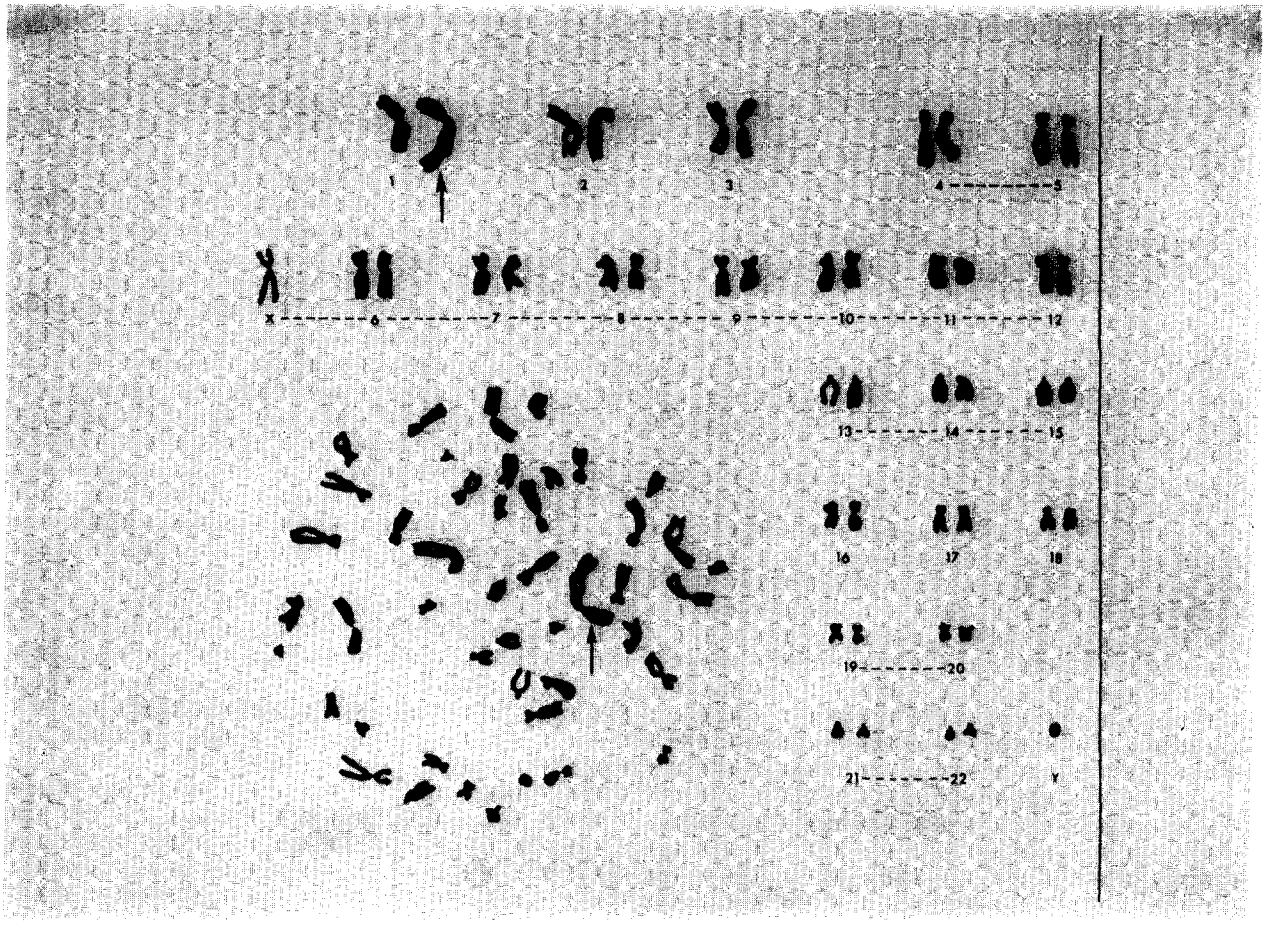


*Fig.* 3. *Karyotype of cell belonging to the major clone* (46, *XY*, 1*q*+) *in Case No.* 58.

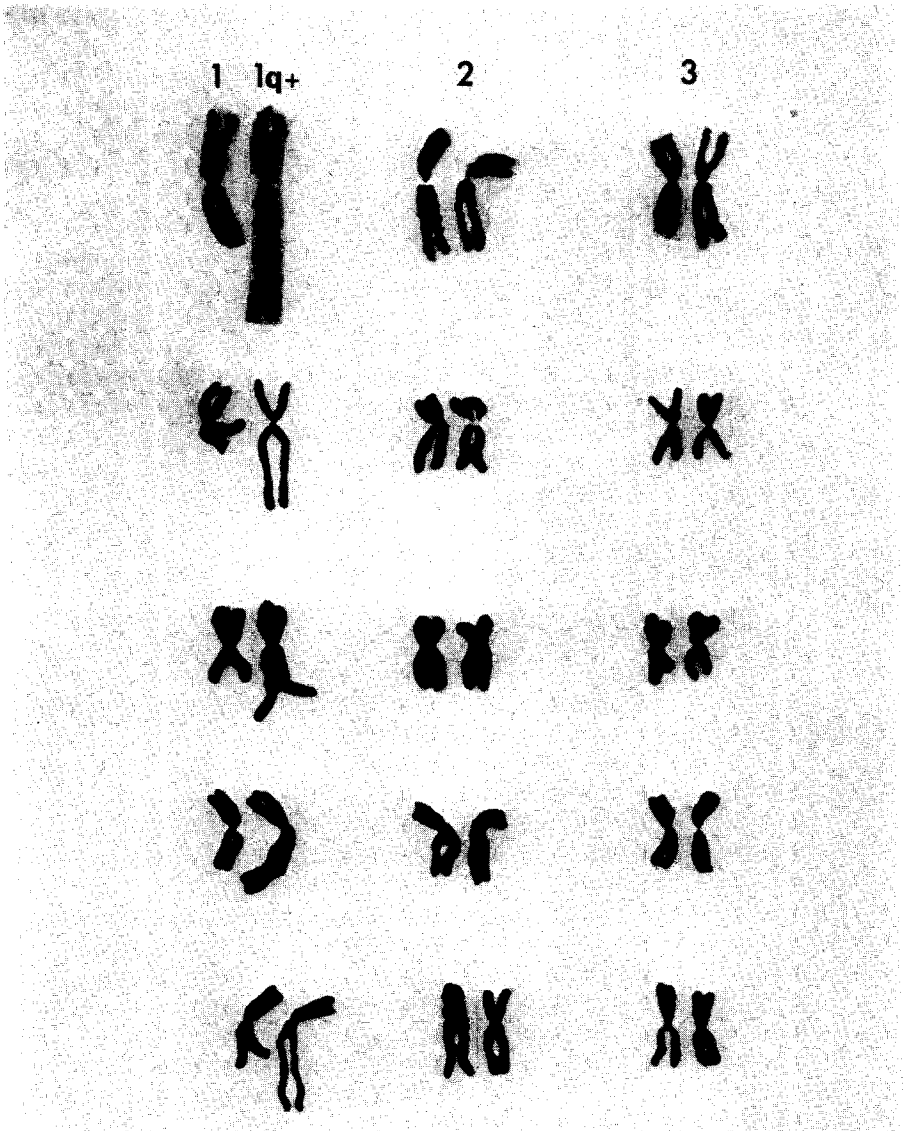


*Fig.* 4. *The marker chromosome* (1q+) *in five cells of the major clone in Case No.* 58.

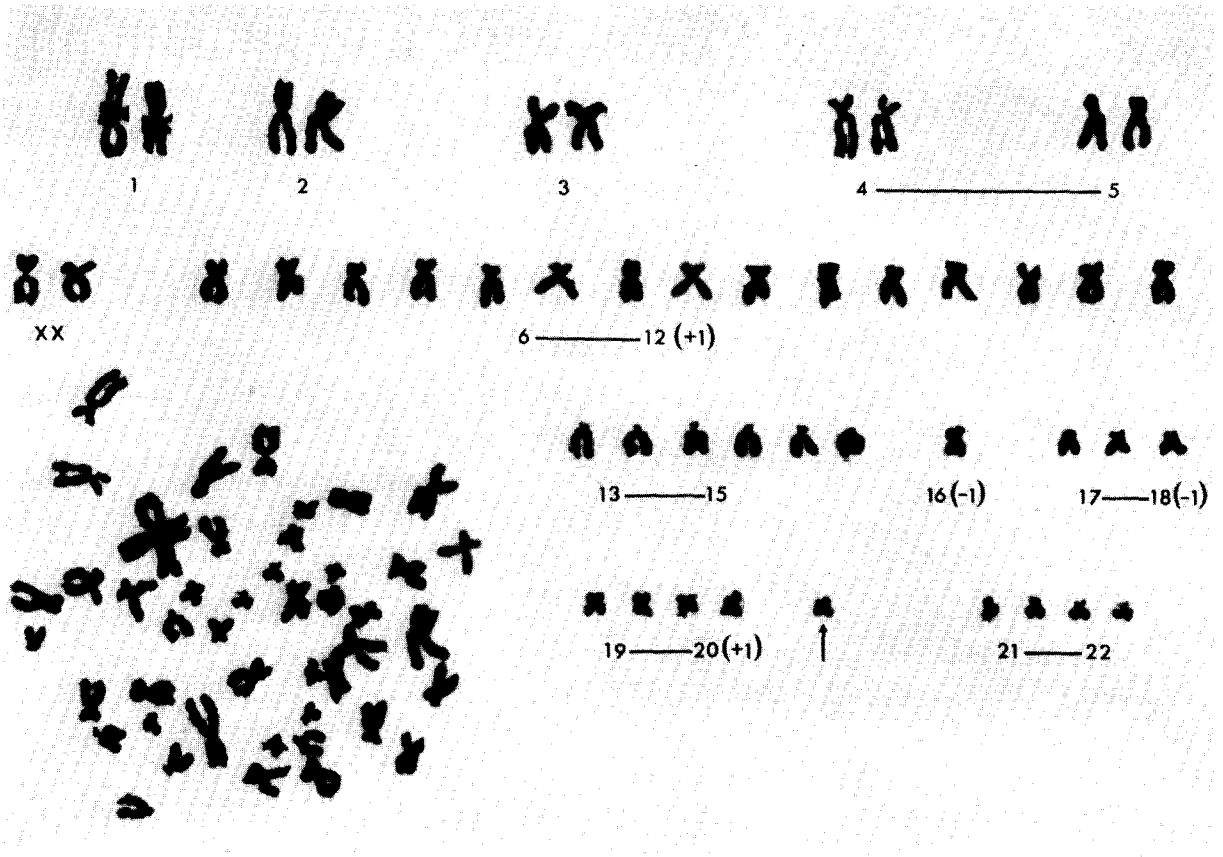


*Fig.* 5. *Karyotype of a cell with a balanced translocation from Case No.* 61.

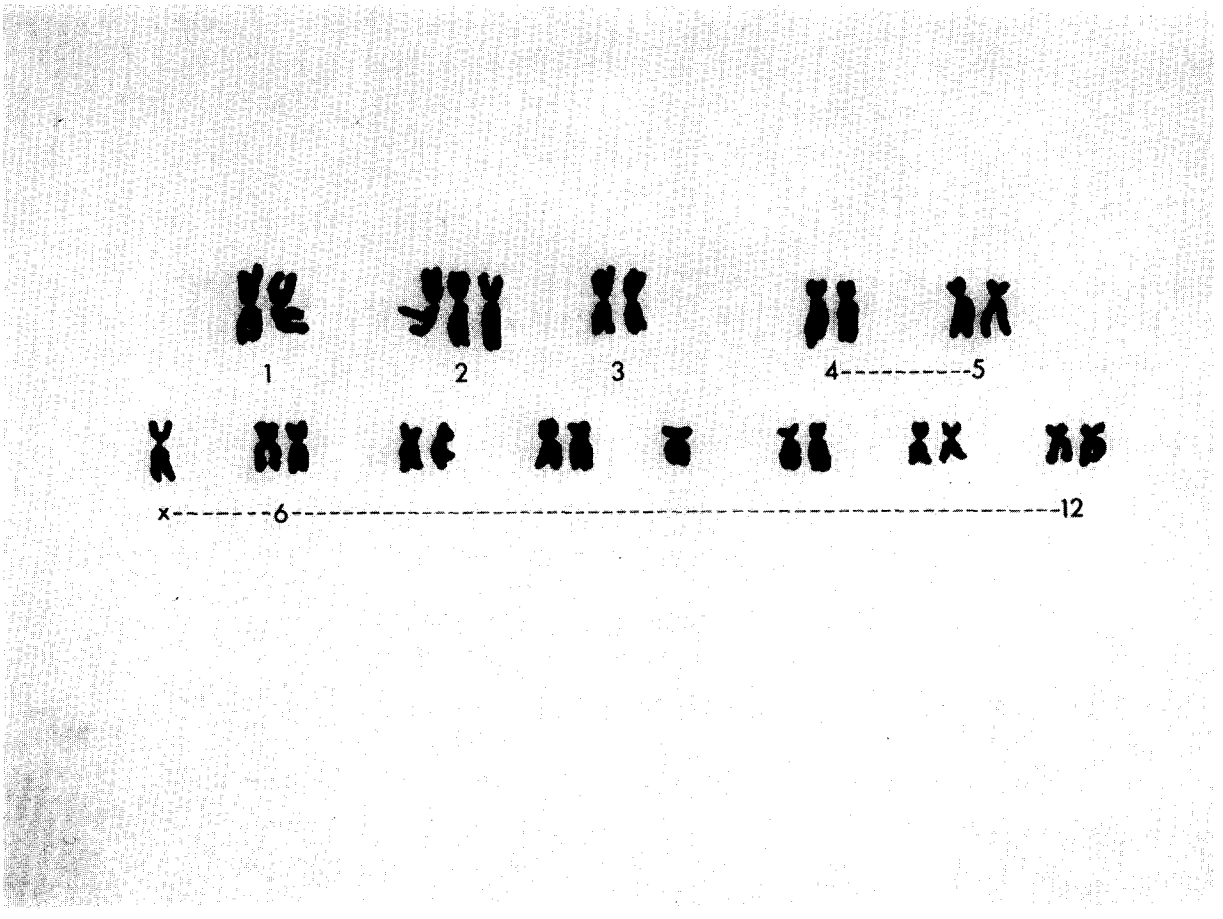


*Fig.* 7. *Chromosomes of groups A, B and C from a cell belonging to a minor clone* (46, *XY*, 2+, *C*−) *in Case No.* 58.

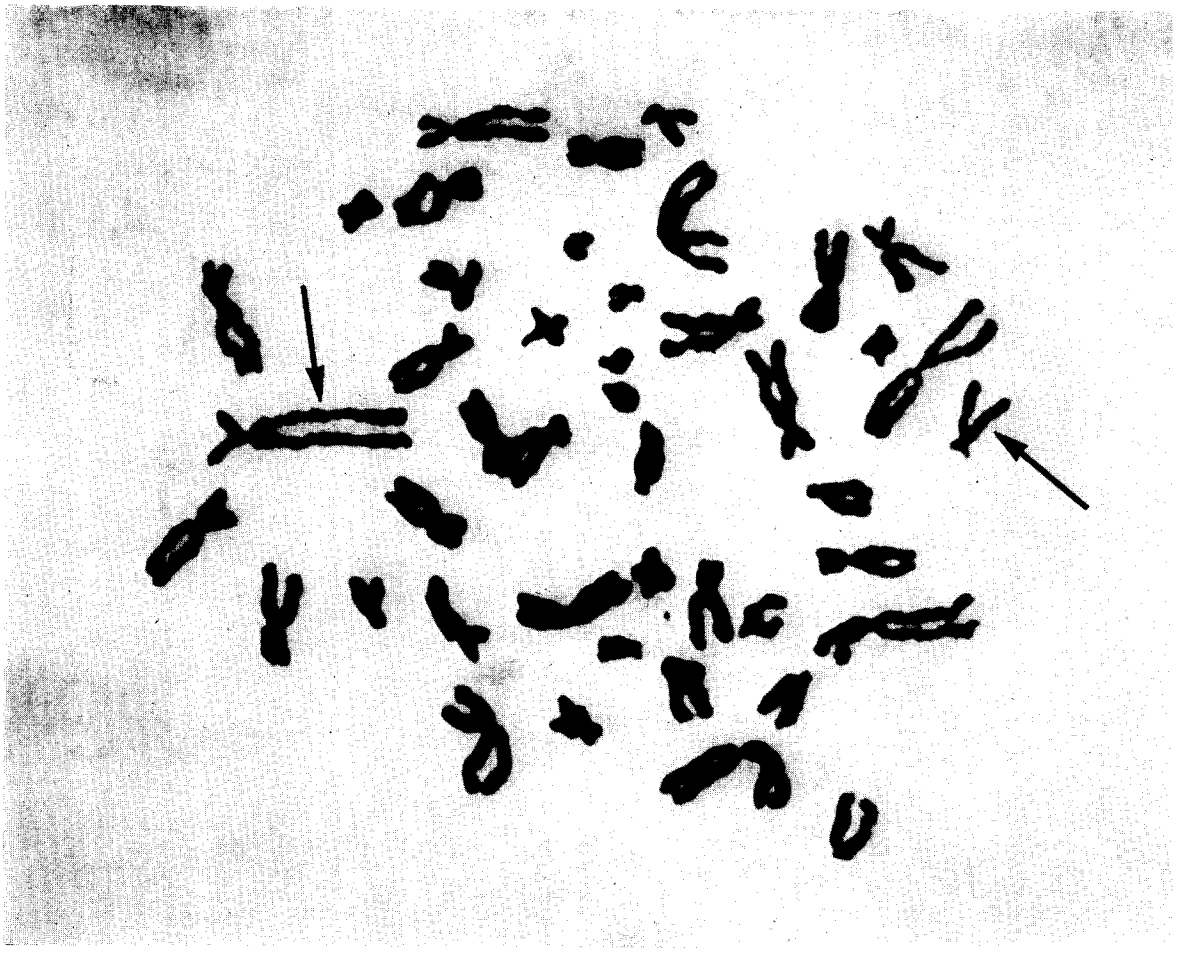

*Fig.* 8. *The marker chromosome in Case No.* 77.

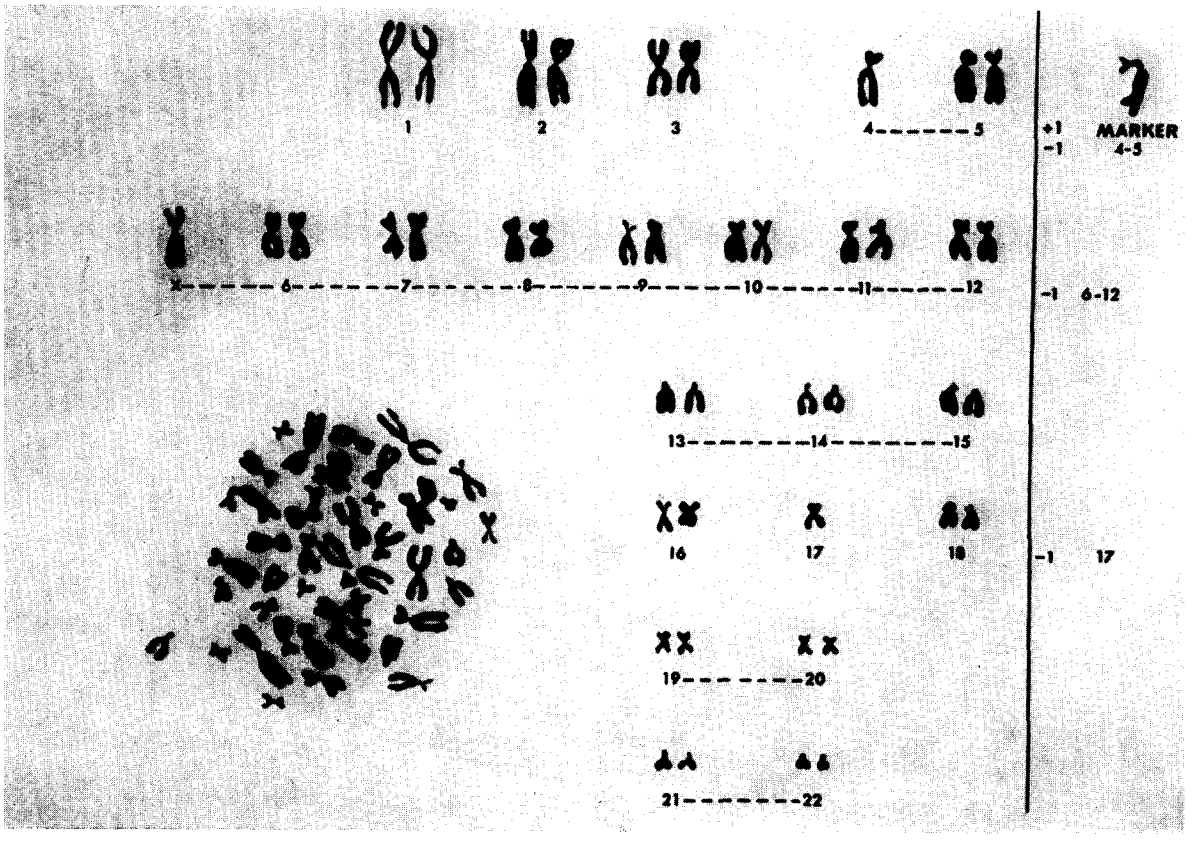


*Fig.* 9. *Karyotype of cell with marker chromosome from Case No.* 31.

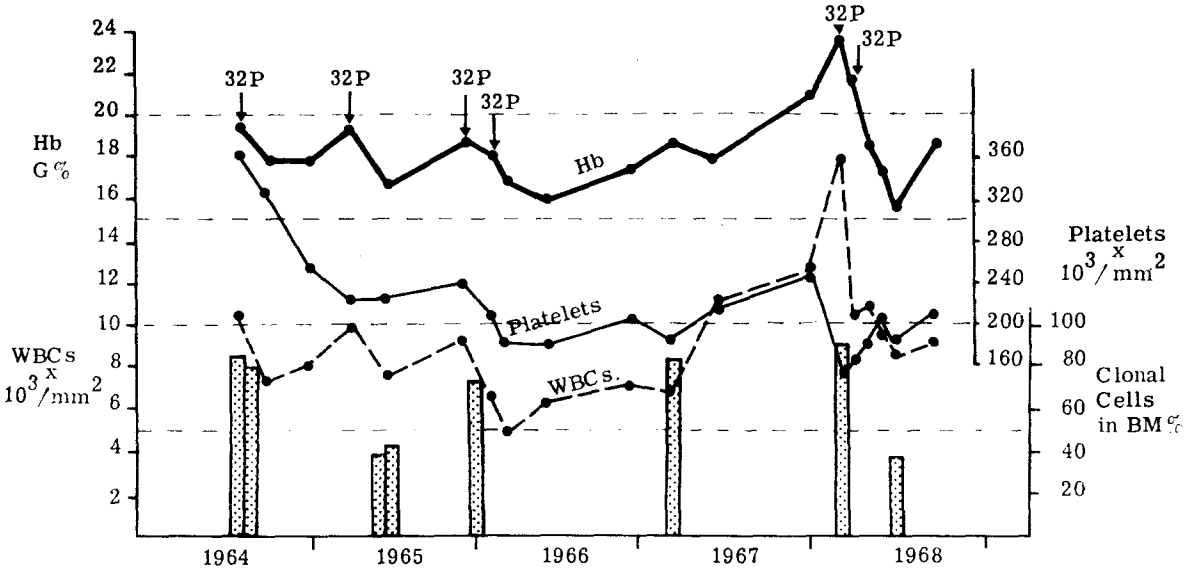


*Fig. 6. The relationship of the abnormal clone (46, XY, Cq−) to blood levels in Case No. 34.*

*Table 5. Serial B.M. aspirates in a patient with a clone of 48, XX, Dq− cells (Case 35)*

| Date | Total $^{32}$P | Last dose $^{32}$P and interval | Hb (g/100 ml) | WBC (mm$^3$) | Plats. (mm$^3$) | Chrososomes of analysed cells: Normal | Clone | Other | Total no. of diploid cells |
|---|---|---|---|---|---|---|---|---|---|
| October 1964 | 16 mCi+ H.V.T. | 5 mCi 16 mths. | 15·3 | 7100 | 66,000 | 19 | 9 | 1. 46, XX, Dq−, Dq+ | 29 |
| November 1966 | 16 mCi | 5 mCi 3¼ yr. | 20·4 | 10,700 | 237,000 | 9 | 30 | 0 | 39 |
| February 1968 | 26 mCi | 5 mCi 1 mth. | 20·8 | 7300 | 171,000 | 6 +(2*) | 26 | 2. 46, XX, 2Dq−, Dq+ | 34 |
| June 1968 | 26 mCi | 5 mCi 5 mths. | 15·2 | 9700 | 270,000 | 5 | 11 | 0 | 16 |
| June 1969 | 30·6 mCi | 4·6 mCi 8 mths. | 17·8 | 8700 | 194,000 | 4 | 32 +(1*) | 0 | 36 |

*polyploid cells.

*Table 6. Serial studies in a patient with major and minor clones (case 58)*

| Date | Hb g/100 ml | WBC (mm$^3$) | Plats. (mm$^3$) | Bone marrow | Chroromosomes of diploid cells: Normal | Major clone (1q+) | Minor clones | Other abnormality | Total |
|---|---|---|---|---|---|---|---|---|---|
| August 1966 | 21·5 | 20,000 | 2,000,000 | Hyperplasia of all series | 36 | 5 | 0 | 2 | 43 |
| August 1967 | 17·2 | 7800 (1%myel-ocytes) | 'plentiful' | Leucopoiesis abnormal with shift to left, suggestive of leuk-aemic change | 41 | 22 | 4. (Dq−) | 1 | 68 |
| March 1968 | 16·0 | 8900 (4%myel-ocytes) | 919,000 | Same appearance as in August 1967 | 164 | 22 | 3. (Dq−) 6. (2+, C−) | 4 | 199 |

*Table 7. Patients with leukaemia*

| Clinical details | | | Haematological status | | | | | Chromosomes | |
|---|---|---|---|---|---|---|---|---|---|
| Case No. | Age and sex | Total $^{32}P$ in mCi (duration of P.V.) | Hypoplasia | Blasts | Erythroid series | Myeloid series: Early | Myeloid series: Late | Mode and (range) | Abnormality |
| 77 | 72 F | 87·7 (16 yr) | Severe | * | | * | | 44 (43–45) | Gross 2 Markers Fig. 8 |
| 46 | 68 F | 24·5 (24 yr) | Moderate | † | | | | 47 (45–47) | 47, XX, C+, F?− |
| 41 | 64 F | 30·0 (10 yr) | | Bone marrow not available †(PB) | | †(PB) | | (40–46) | Gross 2 Markers |
| 31 | 65 F | 59·0 (14 yr) | | † | | ‡ | | 44 (43–45) | Gross Marker Fig. 9 |
| 42 | 64 M | 24·0 (6½ yr) | | * | † | † | | 44, 45(41–47) | 44, XY, 3−, B− 45, XY, B− |
| 18 | 76 M | 39·0 (12½ yr) | | † | ‡ | | | 46 (45–47) | Gross 2 Markers Bizarre polyploids |
| 43 | 71 M | 60·0 (9 yr) | | † | ‡ | | | 46 (44–49) (60–73) | 46, XY, C−, Cr. Heteroploids Bizarre polyploids |
| 33 | 51 M | 64·0 (15 yr) | | * | | † | ‡ | 46, 47(45–47) | Table 8 Fig. 10 |
| 40 | 68 M | 80·0 (18 yr) | | * | | † | † | 46 | 46, XY, Cq−, F?− |

*relative or slight increase
†increase
‡considerable increase

*Case No.* 33. Will be discussed in the following section.

*Cases presenting with or developing leukaemia during the period of observation*

Four patients (Nos. 41, 42, 43 and 77) were already in a leukaemic state with gross chromosomal abnormalities when first studied. [Category (e).] In addition 5 other patients became leukaemic during the period of obsertion. These 5 patients had all been treated and were distributed on first examination in the categories previously listed as follows:

(a) Normal chromosomes — Case No. 18.

(b) Occasional cells showing radiation damage — Case No. 31.

(c) Clones with F?− abnormality — Cases Nos. 40 and 46.

(d) Other major clonal abnormalities — Case No. 33.

These 9 patients form a heterogeneous group according to both the haematological and the chromosomal findings. In Table 7 an attempt has been made to assess the final haematological status. Some idea of the relative frequencies of differentiated and undifferentiated cells in the bone marrow has been given, but it was not possible to summarize the degree of morphological abnormality and of ineffective haemopoiesis which were often the most conspicuous features. The duration of the polycythemia, the total dose of $^{32}P$ and a summary of the chromosomal findings are also given in Table 7. Cases Nos. 77, 42 and 33 had been treated with busulphan as well as $^{32}P$. A brief description of each patient together with a diagnosis based on the haematological features shown in Table 7 follows.

*Case No.* 77. Presented with atypical acute myeloid leukaemia. Hypodiploid cells with two marker chromosomes were found in the bone marrow. A cell is illustrated in Fig. 8.

*Case No.* 46. When first observed this patient had a major clone of cells with the F?− chromosome. Nine months later blasts appeared in the bone marrow accompanied by the presence of a new cell line 47, C+, F?− which became more predominant as the stem cell leukaemia progressed.

*Case No.* 41. Had acute leukaemia and blood culture

*Table 8. Serial Observations in Case 33 showing the distribution of karyotypic variants (minor variants in brackets)*

| Clonal No. | Karyotype | B.M. 13.5.64 | P.B.C. 48–72 hr (no PHA) 15.2.66 | P.B.C. 72 hr (no PHA) 23.5.66 | B.M. 15.7.66 |
|---|---|---|---|---|---|
| Normal | 46, XY | 2 | | ?1 | |
| | (46, XY, C+, D−) | 1 | | | |
| I | 46, XY, Dq− | 8 | 3 | 2 | |
| | (47, XY, G+, Dq−) | | 1 | | |
| II | 47, XY, 2+, Dq− | | 8 | 3 | 4 |
| | (48, XY, A+, G+, Dq−) | | 2 | | |
| III | 46, XY, 2+, C−, Dq− | | 3 | 1 | 3 |
| IV | 47, XY, 2+, C−, 18+, Dq− | | 4 | 8 | 1 |
| V | 47, XY, 2+, C−, 16+, Dq− | | | 2 | 2 |
| VI | 46, XY, 2+, C−2, 16+, Dq− | | | 1 | 4 |
| VII | 46, XY, 2−, C−, Dq−, 18q+ | | | | 1 |
| VIII | As for VII with variable losses | | | | 5 |
| | Total No. of cells | 11 | 21 | 18 | 20 |

showed hypodiploid cells with marker chromosomes. A bone marrow sample was not available.

*Case No.* 31. Showed sporadic abnormalities when first examined. A year later the patient developed acute leukaemia associated with the presence of hypodiploid cells and a long marker chromosome. A karyotype is shown in Fig. 9.

*Case No.* 42. Was anaemic and showed autoimmune disorders. The bone marrow cells were hypodiploid showing a preferential loss of A- and B-group chromosomes.

*Case No.* 18. Had normal chromosomes when first examined. When investigated 17 months later, the patient had erythroleukaemia and the chromosomal analysis of the marrow showed normal cells, bizarre polyploid cells and rearranged cells in the diploid range. Two marker chromosomes were identified.

*Case No.* 43. Presented with erythroleukaemia with a ring chromosome in the majority of cells, the number of chromosomes ranging from 44–49. As the disease progressed the cells with 60–73 chromosomes came to predominate.

*Case No.* 33. This patient originally had a major clone of cells 46, XY, Dq− (see Table 4). Over a period of two years he progressed into a chronic leukaemic state. This progression was accompanied by a series of karyo-

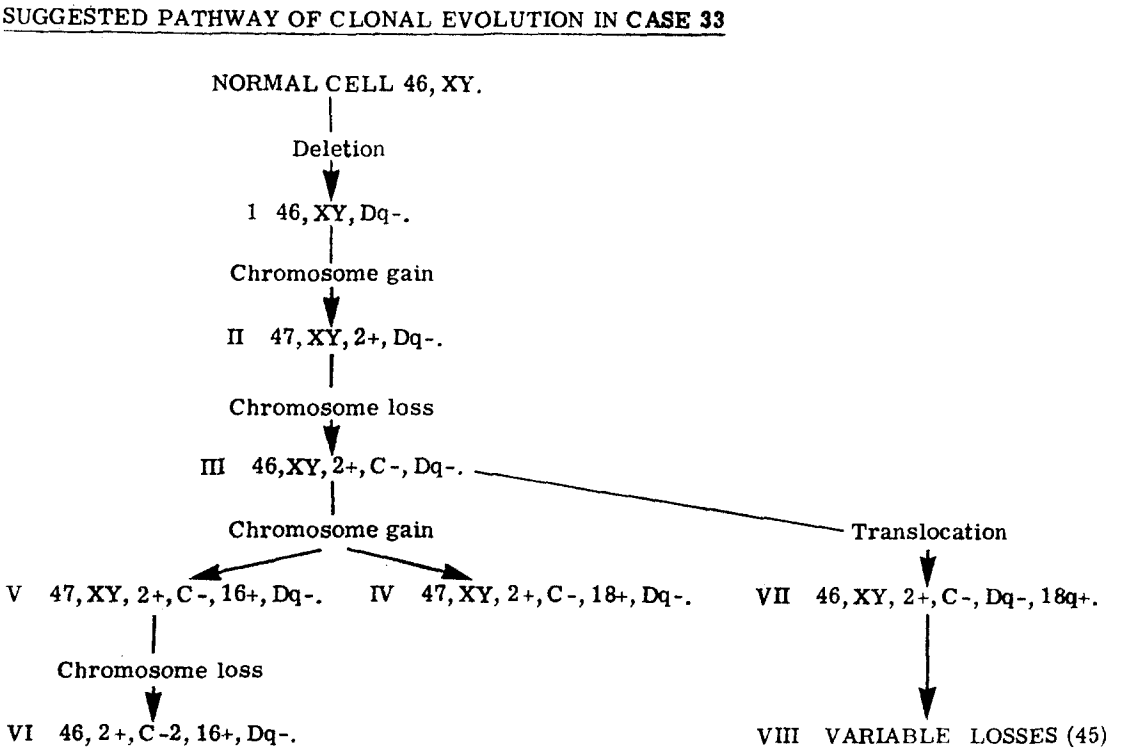


*Fig. 10. Suggested pathway of clonal evolution in Case No. 33.*

typic variations. The karyotypic variants observed are listed in Table 8, and a suggested pathway for the clonal evolution is illustrated in Fig. 10.

*Case No.* 40. Had 46, XY, F?− cells when first examined. One year later he developed atypical chronic granulocytic leukaemia and the bone marrow still contained 46, XY, F?− cells together with 46, XY, Cq−, F?− cells.

## DISCUSSION

*I. Detectability of clones of cells showing the same chromosomal abnormality.*

It is important to realize that clones can only be detected if the cells are dividing; a dormant clone will remain undetected. The recognition of the existence of proliferating clones depends on the extent of the karyotypic derangement—which is related to the problem of resolution — and on the frequency of the clonal cells, the detection of small clones being dependent on the number of cells analysed.

1. *Resolution.* Some of the chromosomal changes induced by $^{32}P$ must be below the limits of resolution. Only relatively large structural rearrangements can be detected by the present crude methods of observation. In a preparation of good quality, for a deletion to be detectable with certainty wherever it occurred in the chromosomal complement, we estimate that the length of missing material must be three times that lost in the formation of the Ph' chromosome. On theoretical grounds it might be expected that the yield of translocations from the low dose-rate β-radiation of $^{32}P$ (<1 rad/hr) [4] would be small and yet sporadic cells with translocations were found in many of the treated cases. Only one of the treated cases with clones (Table 4, No. 61) showed an obvious reciprocal translocation. Apparent deletions were observed in Cases Nos. 33, 34 and 35 but the possibility that the missing material had been translocated elsewhere could not be excluded. The origin of the extra material of the 1q+ chromosome marking the major abnormal clone in Case No. 58 could not be determined.

2. *Size of clone.* At the beginning of the survey we aimed to analyse thirty cells from each bone marrow aspirate. There is no doubt that the analysis of a larger number of cells can reveal the presence of small clones, as, for example, the two minor clones in Case No. 58 (Table 6). These two clones were karyotypically unrelated to the major clone and to each other and must therefore have arisen by independent events.

Occasional cells showing radiation damage were found in many of the patients. The significance of a single rearranged cell in one aspirate cannot be determined, but its presence in a subsequent analysis may be taken as evidence of the existence of a clone as, for example, in Case No. 3 Table 1, the cells with the formula 49, XX, C+2, Mar 1+. If, on the other hand, a similar abnormality can arise on several occasions then such cells would not represent a definite stem line. The subject was discussed by Court Brown *et al.* [5] who examined the chromosomes of blood lymphocytes in irradiated persons. They concluded that the more complex the recurrent chromosome rearrangement, the more likely it is to represent a clone.

*II. Genesis of clones*

The genesis of a detectable clone depends on two separate events, the initial deviation from the normal karyotype (presumably occurring in a single cell), followed by cell selection and a period of proliferation. The initial event apparently differs in the untreated and in the treated group.

1. *Clones in untreated patients.* Clones of aneuploid cells in untreated patients with P.V., were detected in five out of the thirty-three cases examined. The ultimate cause is unknown. In the cases with hyperdiploidy the cells probably originated through non-disjunction of one or more chromosomes of the C-group; there is no evidence whether or not an X chromosome is involved. In one case the Y chromosome was lost, presumably by a similar type of mitotic error.

The clones of aneuploid cells were confined to the bone marrow and involved the polyploid megakaryocytes as well as the diploid granulocytic and erythropoietic cells. Blood lymphocytes and skin fibroblasts had normal karyotypes. Some control data are available on the chromosomal constitution of the bone marrow in haematologically normal persons; in an analysis of 48 females and 32 males no cases with aneuploidy were observed [6]*. It could be that in untreated P.V. the occurrence of random mitotic accidents with the production of aneuploid cells is related to the intense bone marrow hyperplasia. However, the fact that three of the cases with hyperdiploidy had cells with more than one extra chromosome could be in favour of an inherent tendency towards non-disjunction in this disease. In general the aneuploidy was reduced by treatment, but there was one example (Case No. 3, Table 1) of an increase in aneuploidy after therapy.

*In another series, 4 out of 32 males showed a mino population of 45,X,Y− cells (7).

2. *Clones in treated patients.* The F?— abnormality is important for several reasons. Since it was found in ten of the 79 cases examined it has some degree of association with P.V. The chance of the anomaly occurring appears to increase with the duration of the disease. Once F?— cells arise they tend to supplant the normal cells in the bone marrow. The question arises whether or not the F?— abnormality in P.V. is radiation-induced. In many of the cells one whole F-group chromosome appeared to be smaller than the other chromosomes of the group (Fig. 2), and this is not typical of a simple radiation-induced deletion. Moreover, an apparently similar anomaly has been reported in non-irradiated patients with idiopathic sideroblastic anaemia [8]. Only one of our cases with F?— clones (No. 54) had not been irradiated, but she had been treated with busulphan. Therefore, we cannot exclude the possibility that in P.V. the anomaly is related to treatment.

Apart from the F?— abnormality the commonest type of acquired clonal abnormalities were simple deletions or translocations. These abnormalities were also seen in the polyploid cells in most cases, so presumably the megakaryocytic series was also involved. These clones, seen only in treated patients, are presumably radiation-induced since all the cases with clones had received substantial amounts of $^{32}P$ (Table 4). Nowell and Hungerford [9] have described similar clones in several patients with P.V. treated with $^{32}P$. One other group of irradiated persons in whom the chromosomes of the bone marrow have been examined is the Japanese fishermen who were exposed to fall-out radiation [10]. It was of some interest that 12–13 years after the incident, clones of abnormal cells were detected in the bone marrow, but not in the blood lymphocytes in several men. In our series also, there was no evidence of clones of chromosomally abnormal cells in blood lymphocyte cultures.

*III. Selective factors*

1. *Homeostasis.* The propagation of clones within the bone marrow may be influenced by substances such as erythropoietin that normally exert homeostatic control over blood cell formation. Some evidence that the disease may be due to a clone of cells insensitive to regulatory controls is provided by the situation found in Case No. 8 (Table 2). In this case the fluctuations of the karyotypically normal and the aneuploid 45,X,Y— cells coincided with the blood levels of haemoglobin and platelets, and, to a lesser extent, of leucocytes. When these levels were high, the normal cells appeared to be totally inhibited from proliferation whilst the abnormal clone was not.

A similar situation appertained in cases 34 (Fig. 6) and 35 (Table 5) although in both these cases the clones as recognizable by chromosome analysis were probably radiation-induced. The karyotypically normal cells in Case No. 34 were never totally suppressed, perhaps because the high haemoglobin levels and leucocyte counts were not accompanied by thrombocytosis (Fig. 6).

2. *Other factors.* In some cases with clones showing radiation-induced chromosomal abnormalities it is possible that the cells carry a proliferative advantage which may not be directly related to the recognizable chromosome change. Such a selective advantage of chromosomally marked populations of cells has been demonstrated in heavily irradiated mice. [11–13].

On the other hand the unique behaviour of the F?— clone, its incidence (10 out of 79 cases) and progressive increase in most patients may be a reflection of a proliferative advantage that is causally related to the chromosomal anomaly.

The influence of the degree of radiosensitivity on the behaviour of clones must also be considered. In this respect the success of the F?— clone might be attributed to the development of radioresistance in some cases, for example 37 and 48.

*IV. Leukaemia*

The nine patients showing haematological evidence of leukaemia (Table 7) all had abnormal chromosomes in bone marrow cells but the karyotypic rearrangements were unique to each patient. All were long standing cases of P.V., the mean duration of disease being $14\frac{1}{2}$ years, and each of them had received a substantial dose of $^{32}P$. Judging from these and similar reported cases [9, 14, 15] the incidence of chromosomal abnormalities is very high; indeed no case of acute leukaemia following P.V., with normal chromosomes has been recorded. This is in contrast to the situation in adult acute myeloid leukaemia arising *de novo*, where amongst 60 patients [16–18] more than half had no apparent chromosomal abnormalities.

It was of interest that in our series the leukaemic cases, for which chromosomal data were available before leukaemia developed, were drawn from all the various categories, viz. normal chromosomes, occasional cells with

radiation damage, clones with F?— anomaly and major clonal abnormalities. This showed that leukaemia was not more likely to supervene in cases classified in any particular category.

Our cases showed a spectrum of chromosomal changes, varying in karyotypic complexity and in speed of progression. At one end of the spectrum are the gross abnormalities with marker chromosomes (Cases Nos. 77, 41, 31 and 18). It was not possible to trace the karyotypic evolution but in each case at least three structural rearrangements must have occurred. The bone marrow cells of Case No. 18 had a normal karyotype 1½ year previously, and Case No. 31 had predominately normal cells with occasional cells showing radiation damage one year before leukaemia developed. The two cases of erythroleukaemia, 18 and 43, had bizarre polyploid cells and, in Case No. 43, abnormal heteroploid cells with 60–73 chromosomes eventually predominated.

At the other end of the spectrum were the cases showing minimal chromosomal changes associated with a slower progression into the leukaemic state and the presence of general bone marrow hypoplasia. These minimal changes are exemplified by Case No. 46, a patient presenting with an F?— clone, the only additional abnormality in the leukaemic state being the gain of a C-group chromosome.

Case No. 33 was the only one in which karyotypic evolution was observed. Over a period of two years, as granulocytic hyperplasia increased, a step-by-step karyotypic evolution could be traced. A suggested pathway of clonal evolution is given in Fig. 10, the majority of changes can be accounted for by non-disjunctional events. New karyotypic variants continued to arise but not one of them established dominance so that in the final bone marrow sample the whole range of intermediate steps was represented. In acute leukaemia supervening in chronic granulocytic leukaemia clonal evolution is observed not infrequently [19–25] and one particular pattern of karyotypic change has been observed in several patients [21, 25]; this is in marked contrast to P.V., where the chromosomal changes observed in each leukaemia patient were unique.

An intermediate but probably exceptional situation is represented by Case No. 58 (Table 6). Cytologically, over a period of 18 months, the bone marrow has suggested incipient leukaemia. Chromosomally, this patient showed a major clone with a marker chromosome and minor clones both related and unrelated to the major one. Despite these ominous signs karyotypically normal cells persist and normal haemopoiesis is maintained in a state of precarious balance.

Extensive karyotypic changes involving chromosomal rearrangements have been reported in many cases of leukaemia with no history of irradiation. In our cases of leukaemia it is improbable that either major structural changes or aneuploidy are the direct consequences of irradiation. There is no evidence from our data to suggest that radiation-induced chromosomal changes, in clones or in single cells, portend the development of leukaemia.

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## SUMMARY

*The significance of chromosomal changes in bone marrow cells in polycythaemia vera has been studied in a series of 79 patients. Chromosomally marked populations of cells were of four types:*

*(a) Aneuploid clones present before treatment commenced.*

*(b) Clones with a similar anomaly of one of the F group chromosomes, F?—, in several patients.*

*(c) Clones with structural alterations probably due to treatment with $^{32}P$.*

*(d) Varied and often complex rearrangements associated with the development of leukaemia.*

*In some cases aneuploid clones and in other cases radiation-induced clones showed fluctuations in proliferative activity which were related to haematological status. The chromosomally marked populations of cells appeared to predominate in relapse when the blood levels were high, suggesting a lack of sensitivity to homeostatic controls.*

*The F?— clones usually showed progressive increase at the expense of the normal population.*

*None of the three types of clone was directly related to the development of leukaemia, nor*

*was there any evidence to suggest that radiation-induced chromosomal changes portend the development of leukaemia.*

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# A Scanning Electron Microscope Study of Cell Surface and Cell Contacts of "Spontaneously" Transformed Cells *in vitro*

GISELE M. HODGES

*Department of Cellular Pathology, Imperial Cancer Research Fund, London, W.C.2, England*

## INTRODUCTION

THE surface properties of cells and intercellular contacts have been implicated in many aspects of cell function and behaviour such as cell movement of normal and malignant cells [1], selective aggregation and sorting out of cells [2], malignant transformation of cells by viruses [3], and embryonic induction [4]. It is also probable that the cell surface may play an important role in carcinogenesis [5].

Transmission electron microscope studies of the surface structure of intact normal and malignant cells have been carried out by various authors either by direct visualization [6, 7] or by the preparation of shadowed surface replicas [8–11]. There are, however, limitations to such studies. Direct visualization of external surface detail is limited by specimen thickness and although better resolution is possible by the use of replicas this technique is time-consuming. Transmission electron microscope studies of ultra-thin sections of cells have resulted in an extensive literature on the structure of the cell membrane [12]. Reconstructions from serial thin sections can be used to elucidate the three-dimensional relationships of the cell components but such methods are laborious. The scanning electron microscope recently introduced into biological research (for review see [13]) has a number of advantages in that specimen preparation is comparatively easy and resolution and the depth of focus is greatly increased compared to that of the optical microscope providing a high magnification three-dimensional perspective of surface structure. Brief descriptions of the scanning electron microscope appearance of tissue-culture cells have been reported by various authors [14–16].

The present report describes the surface appearance and cellular contacts, as seen in the scanning electron microscope, of different transfer generations of mouse kidney cells during the process of "spontaneous" transformation. It forms part of a series in which these changes in young and old mice are being compared [17].

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## MATERIAL AND METHODS

*Cell cultures*

Two cell strains, CBM 17 (derived from $C57_a{}^t$Icrf mice) and CBM 18N (derived from $C_3H$ Bittner mice) were examined. Both cell lines were grown from trypsinised cell suspensions of 3-day old mouse kidneys. CBM 17 and CBM 18N were routinely transferred by trypsinisation. When inoculated into syngeneic mice both strains gave rise to tumours. Full details of these cell lines are reported elsewhere [17].

The following cultures were used in the present study. CBM 17/9 and CBM 17/42

| Culture no. | Transfer generations | Total days in culture |
|---|---|---|
| CBM 17 | 9 | 264 |
| CBM 17 | 42 | 509 |
| CBM 17 | 45 | 417 |
| CBM 18N | 37 | 600 |

were started from frozen stock and required several weeks to become re-established in culture.

For scanning electron microscopy, the cells were cultured on 16 mm coverglasses (Chance) in glass petri dishes until confluent monolayers were just forming. The coverglasses bore a silicone rubber mark (Esco Rubber Ltd. — silicone rubber moulding paste) in order to facilitate identification of the cell-carrying surface.

*Scanning electron microscopy*

Each coverglass was washed several times with Waymouth's MB 752/1 medium (warmed to 37°C) to remove extraneous serum proteins from the cell surface. The cell cultures were then immediately fixed in 2·5% glutaraldehyde in 0·1M sodium cacodylate buffer, pH 7·1 for 2 hr at 4°C. They were subsequently rinsed in four changes of buffer at 4°C for 24 hr. The cultures were dehydrated at 1 hr intervals through a graded series of alcohols at room temperature (30%, 50%, 70%, 95% and 2 changes of absolute alcohol). The coverglasses were either left in absolute alcohol until required for examination or they were allowed to air-dry and stored in a dessicator.

For examination in the scanning electron microscope the coverglasses were mounted on Dural specimen holders coated with an adhesive ("Sellobond" — Sellotape Products Ltd.). An electrically conducting surface coating of gold/palladium alloy (thickness ca. 500 Å) was applied to the coverglasses by vacuum evaporation with rotation of the specimens to ensure uniform coverage of the surface. The metal coating minimizes the charging effects of the electron beam that would otherwise decrease resolution. The specimens were then examined in a scanning electron microscope (Cambridge Scientific Instruments "Stereoscan" Mk IIa) with the angle of incidence of the primary electron beam to the specimen surface at 45°. The photomicrographs were recorded on Ilford 35-mm HP3 film during 40 or 100 second exposures.

## OBSERVATIONS

*Cell surface*

Light microscopy of CBM 17 cell cultures from three different culture generations (9th, 42nd and 45th) did not show any essential differences in morphology. Spindle-shaped fibroblast-like cells forming a meshwork system and pavement-like sheets of cells of a more polygonal shape were the main cell components but very flattened giant cells were also present (Figs. 1 and 2). Junctions between giant cell and surrounding cells were frequently observed without apparent overlapping (Figs. 1 and 2). The giant cells showed considerable variation in size and shape, contained one or more nuclei and possessed a relatively opaque juxtanuclear cytoplasm distinguishable from an extensive peripheral semi-transparent cytoplasm (Fig. 2).

The morphological features seen in living cell cultures were recognizable by scanning electron microscopy but in much greater detail. The cell surface of the giant cell was resolved into the three main cytoplasmic regions—nuclear, juxtanuclear and peripheral zones. The nuclear region, at low magnification, had a smooth textured overlying cytoplasm (Figs. 3 and 4) while at higher magnification numerous shallow depressions could be distinguished. Protrusions of varying size, shape and number were present, frequently associated with small deeper depressions at their base.

The juxtanuclear cytoplasm, characterized by a very rough-textured surface, was more extensive at one pole and varying numbers of large shallow depressions could be observed in this area (Figs. 3 and 4).

The peripheral flattened cytoplasmic region had a smooth-surfaced texture with numerous striations apparently underlying the cell surface (Figs. 3 and 4). These elements extended throughout the length of the cell and varied in diameter from 500 to 1000 Å. Microvilli were rarely seen on the upper free surface of the giant cells but were present along the cell margins.

The meshwork arrangement of the elongate fibroblast-like cells (Fig. 5) and the pavement grouping of the polygonal shaped cells (Fig. 6) were clearly seen with the scanning electron microscope. Fibrils, possibly collagen, extended in various directions over the surface of some of the more confluent cultures (Fig. 7). The surface features of the fibroblast-like cells and

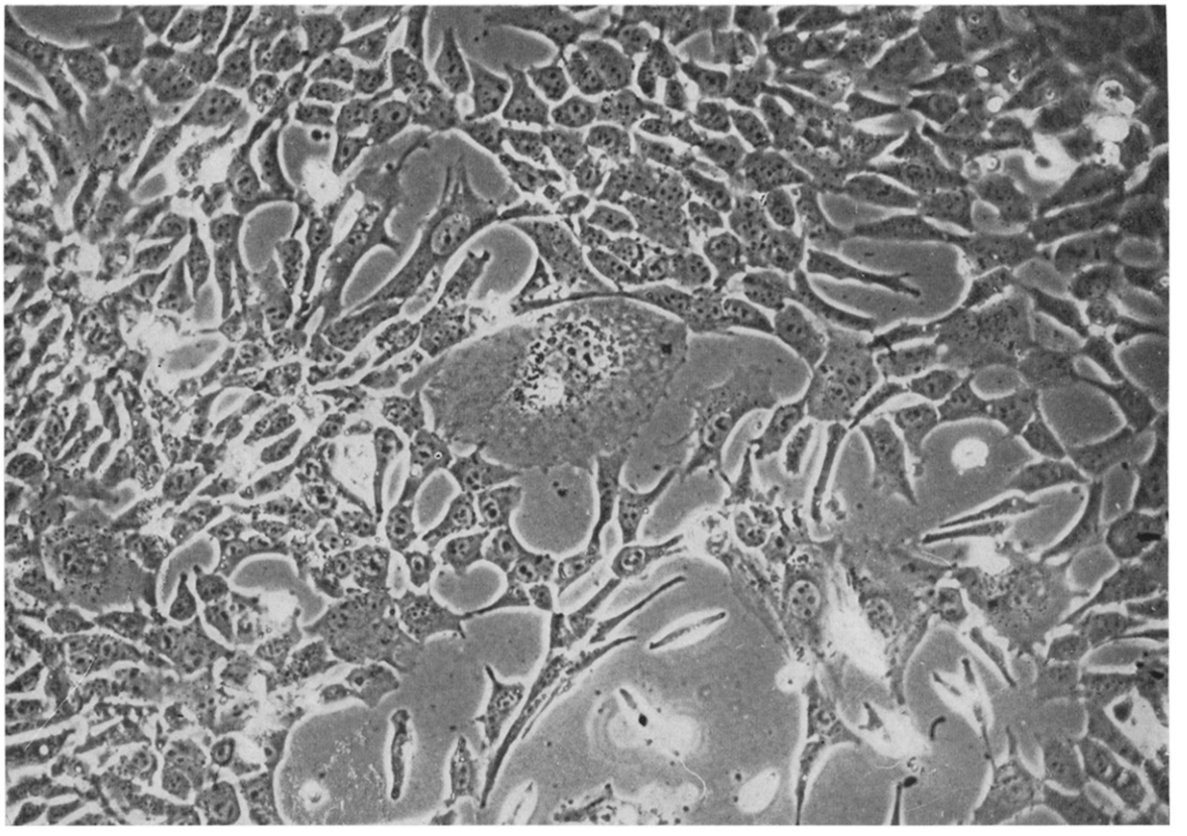

*Fig.* 1. *CBM* 17/42. *Living cell culture showing three main cell types: giant cell, fibroblast-like cell, polygonal-shaped cell. Phase contrast.* ×400.

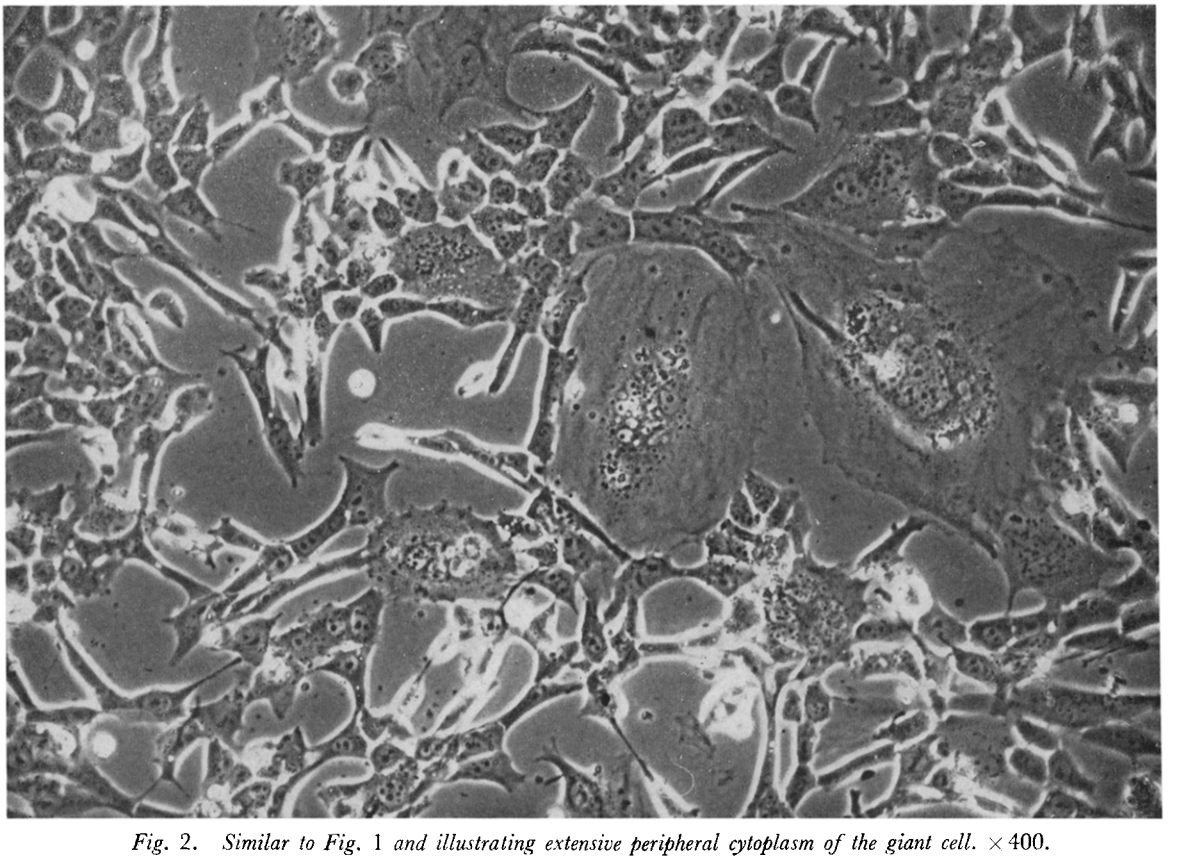

*Fig.* 2. *Similar to Fig.* 1 *and illustrating extensive peripheral cytoplasm of the giant cell.* ×400.

(to face p. 236)

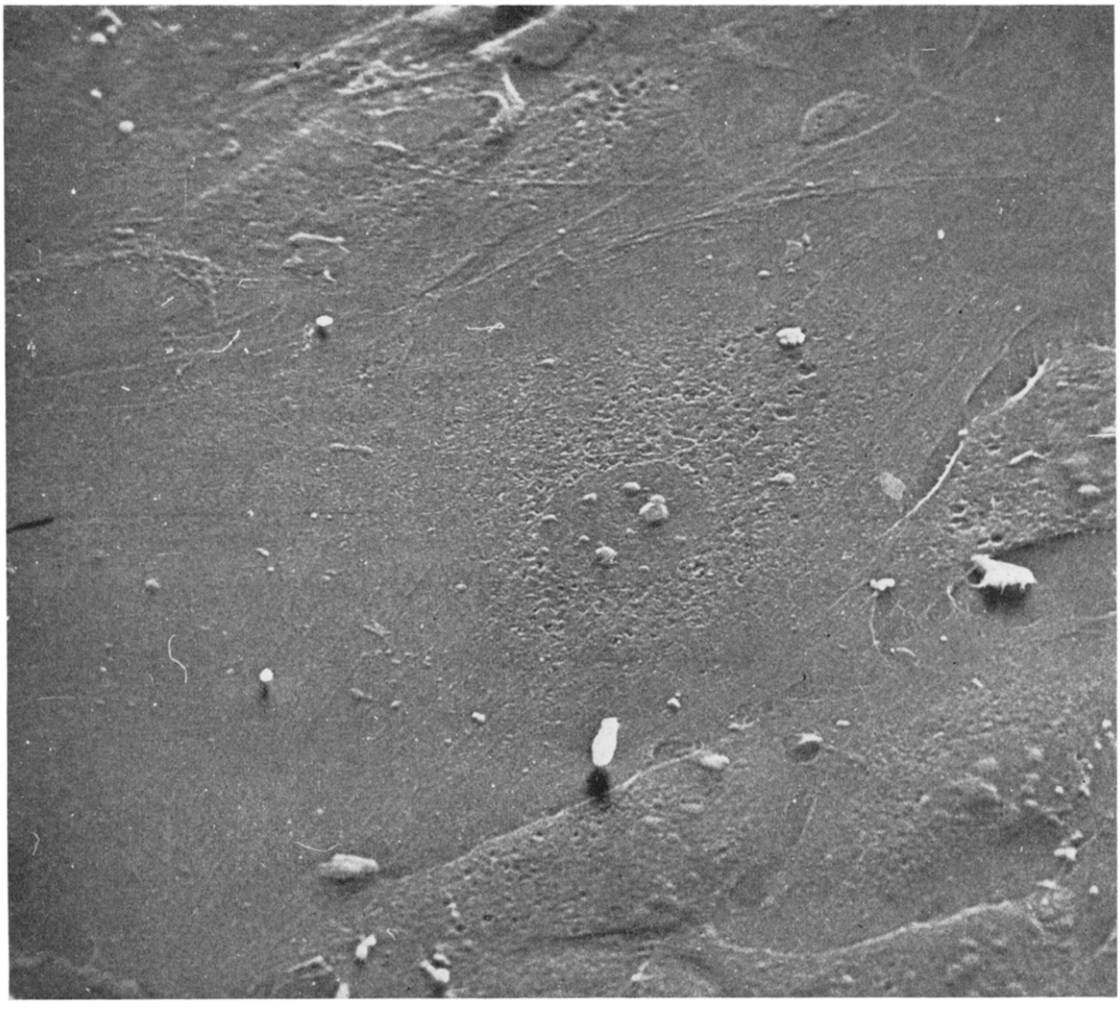

*Fig.* 3. *CBM* 17/9 *culture showing surface topography of giant cell.* $\times 2800$.

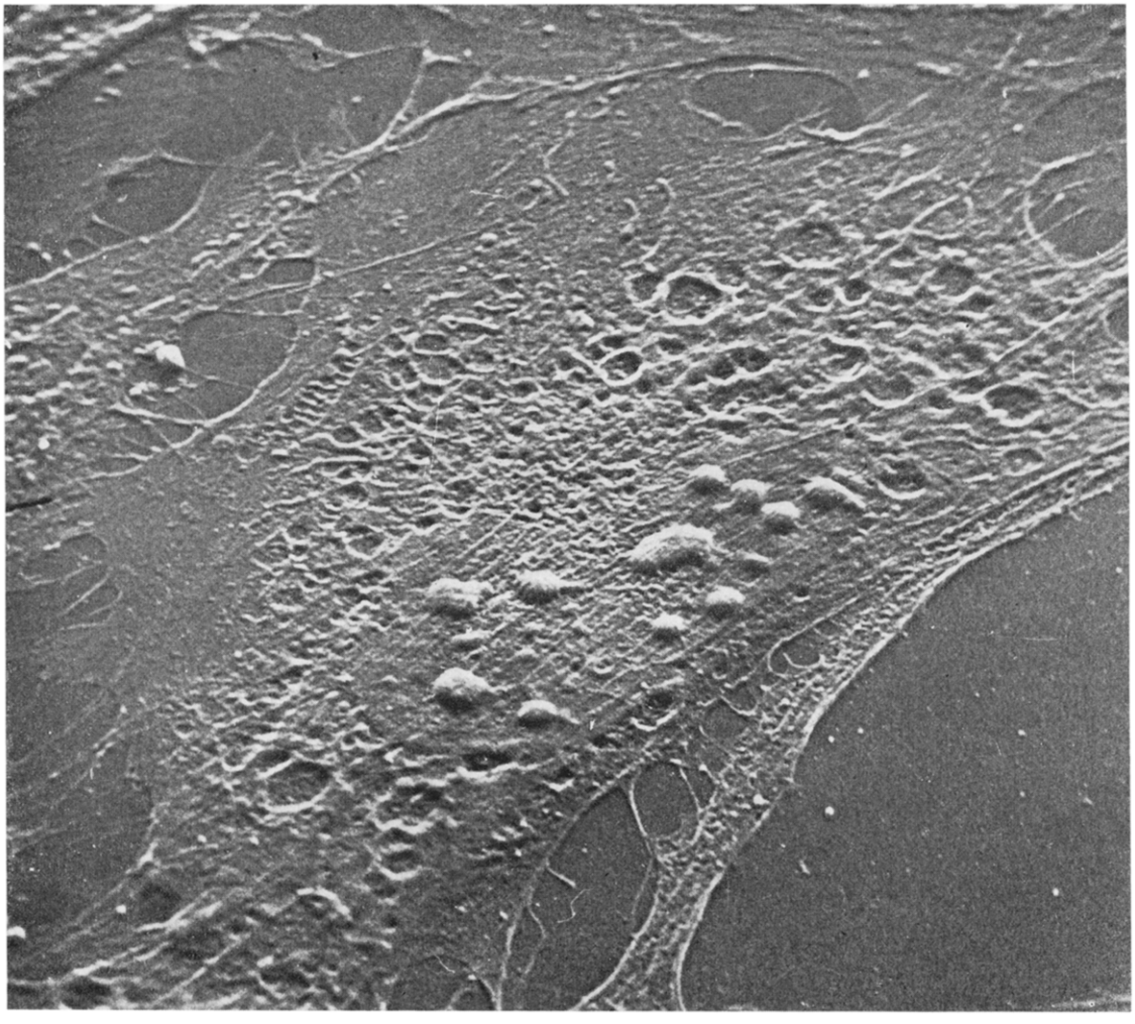

*Fig.* 4. *CBM* 18/37 *culture showing binucleate giant cell.* $\times$ 3900.

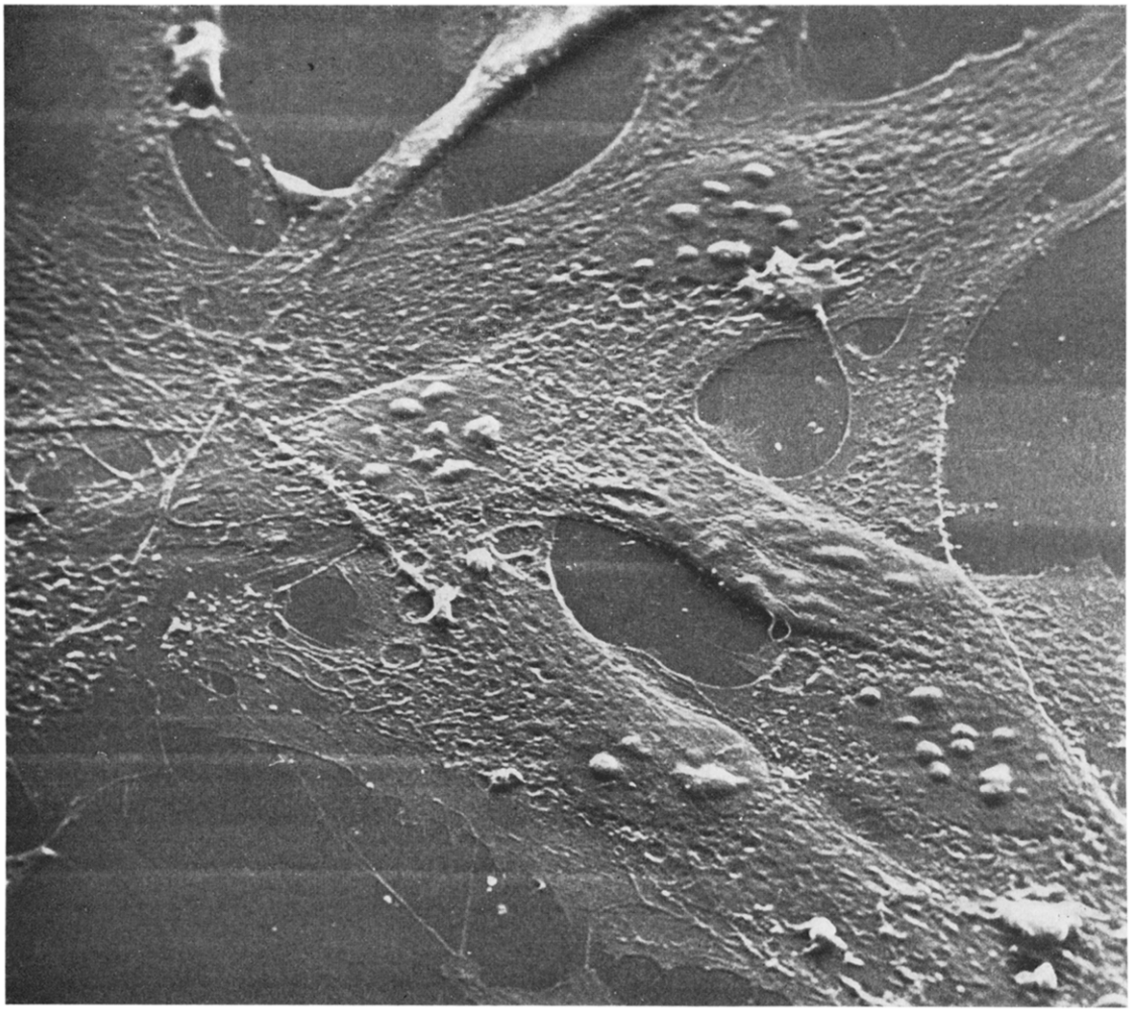

*Fig. 5. CBM 18/37 culture. Meshwork arrangement of fibroblast-like cells, showing overlapping of cells.* $\times 1980$.

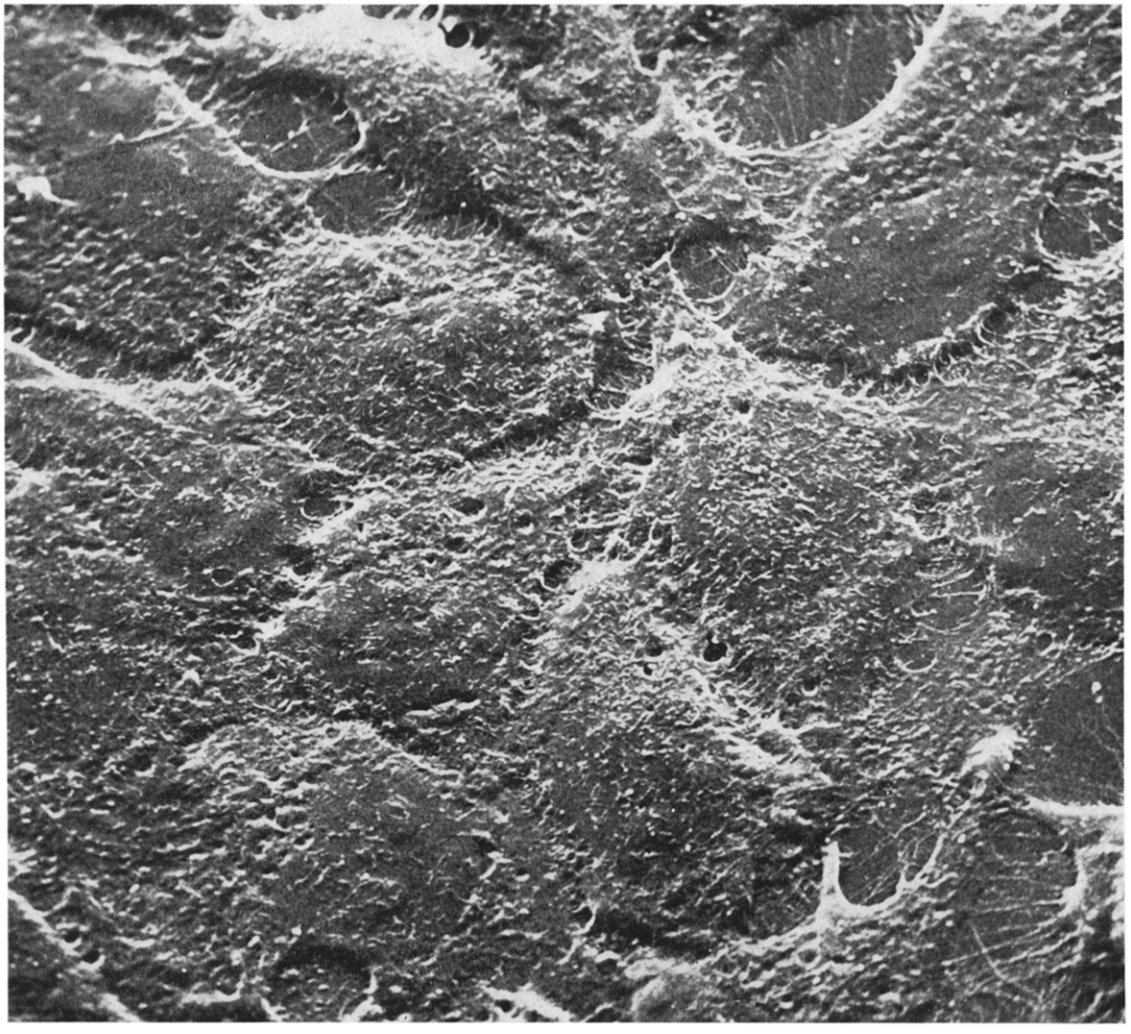

*Fig.* 6. *CBM* 17/42 *culture. Pavement grouping of polygonal-shaped cells, showing lateral apposition of cells.* $\times$ 2800.

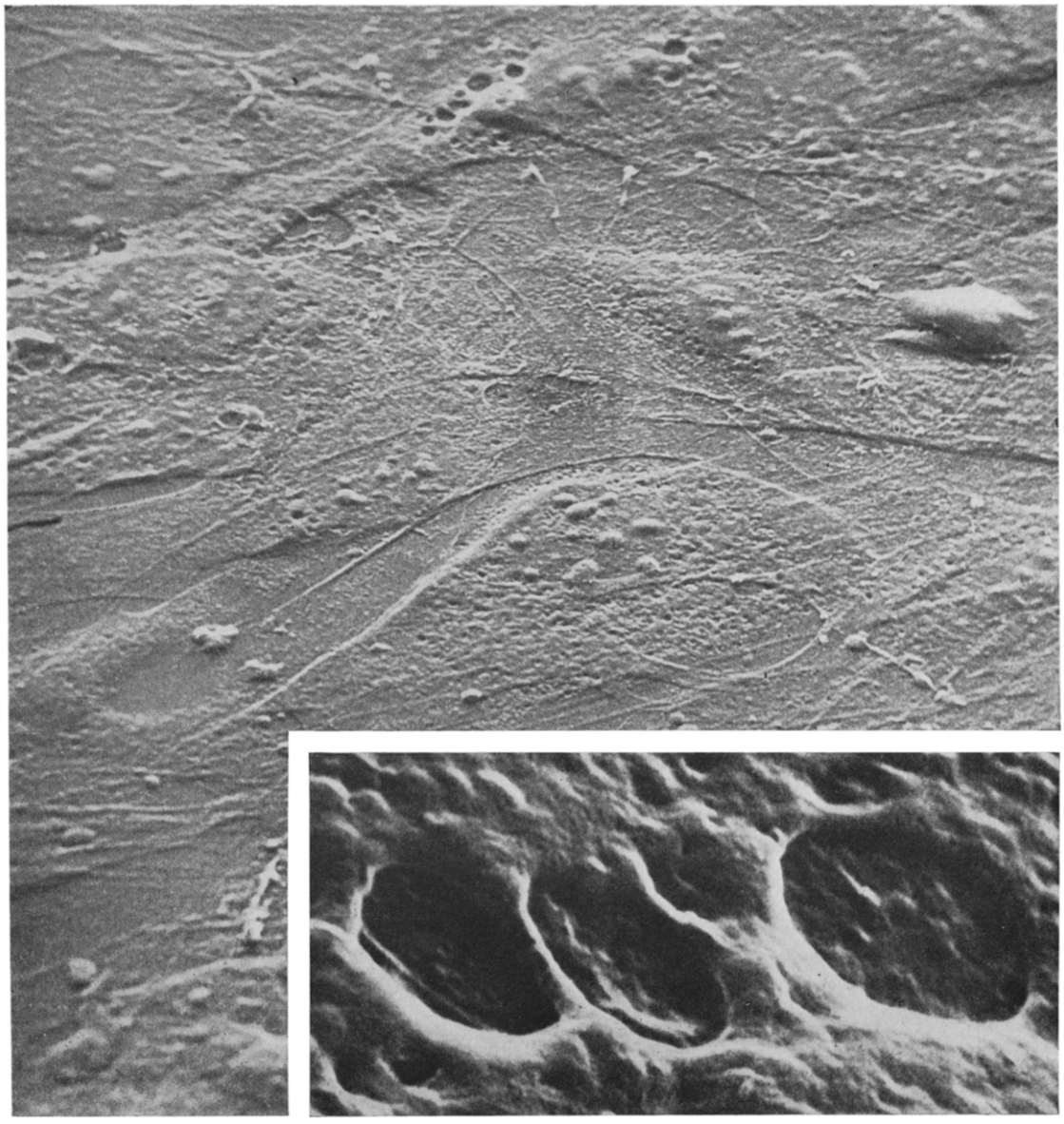

*Fig. 7. CBM 17/9 culture. Confluent culture with fibrils extending over the surface and showing fibroblast-like cell with fenestrae.* × 1900 (*inset* × 1900).

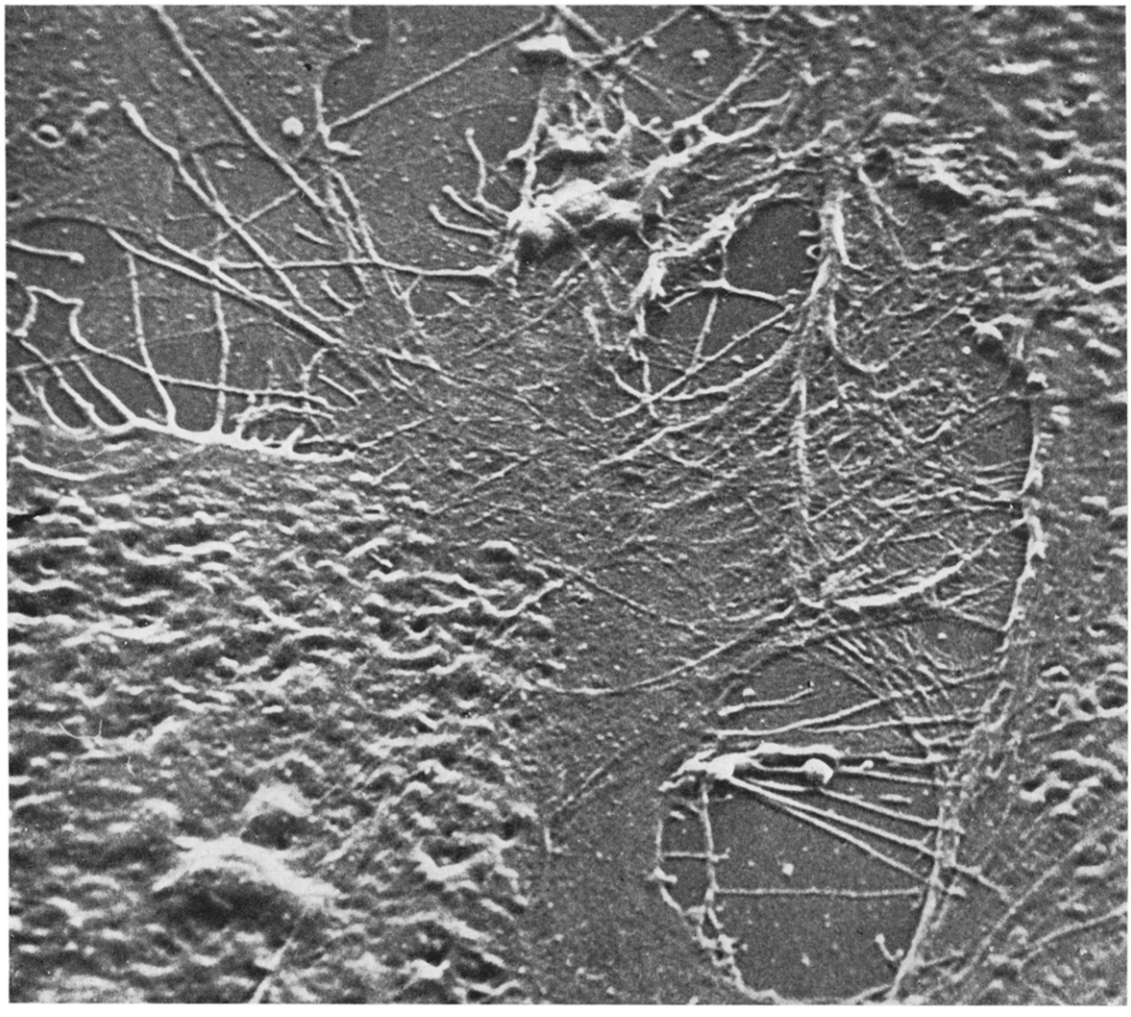

*Fig.* 8. *CBM* 17/9 *culture. Laterally situated microvilli forming complex interdigitations between adjacent cells.* ×9400.

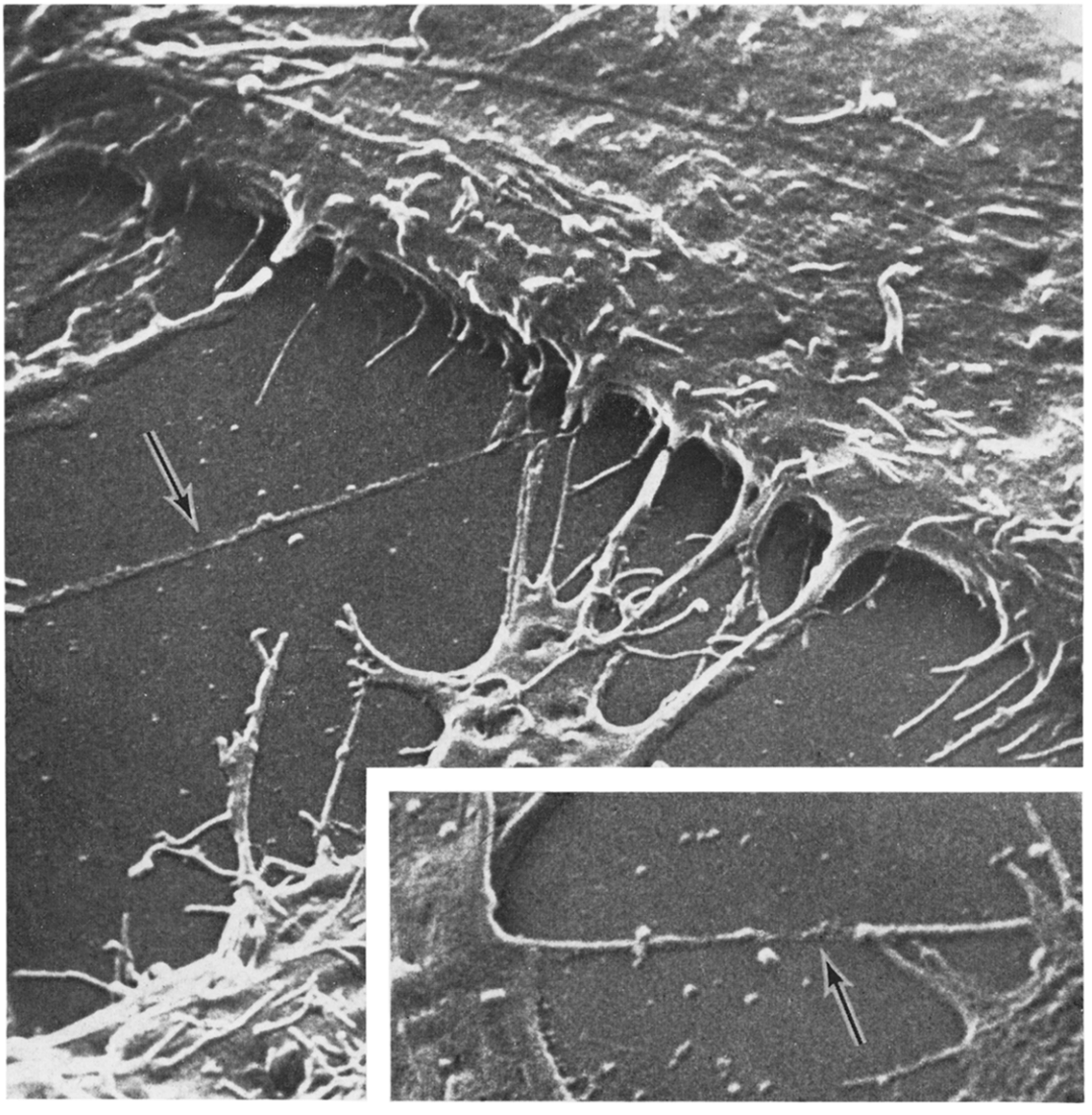

*Fig.* 9. *CBM* 17/42 *culture showing contacts* (*a*) *between microvilli of adjacent cells and* (*b*) *between cells and the underlying glass substratum. The arrows indicate fine threads of cytoplasm remaining adherent to the glass sufrace.* $\times$ 14,400 (*Inset* $\times$ 18,400).

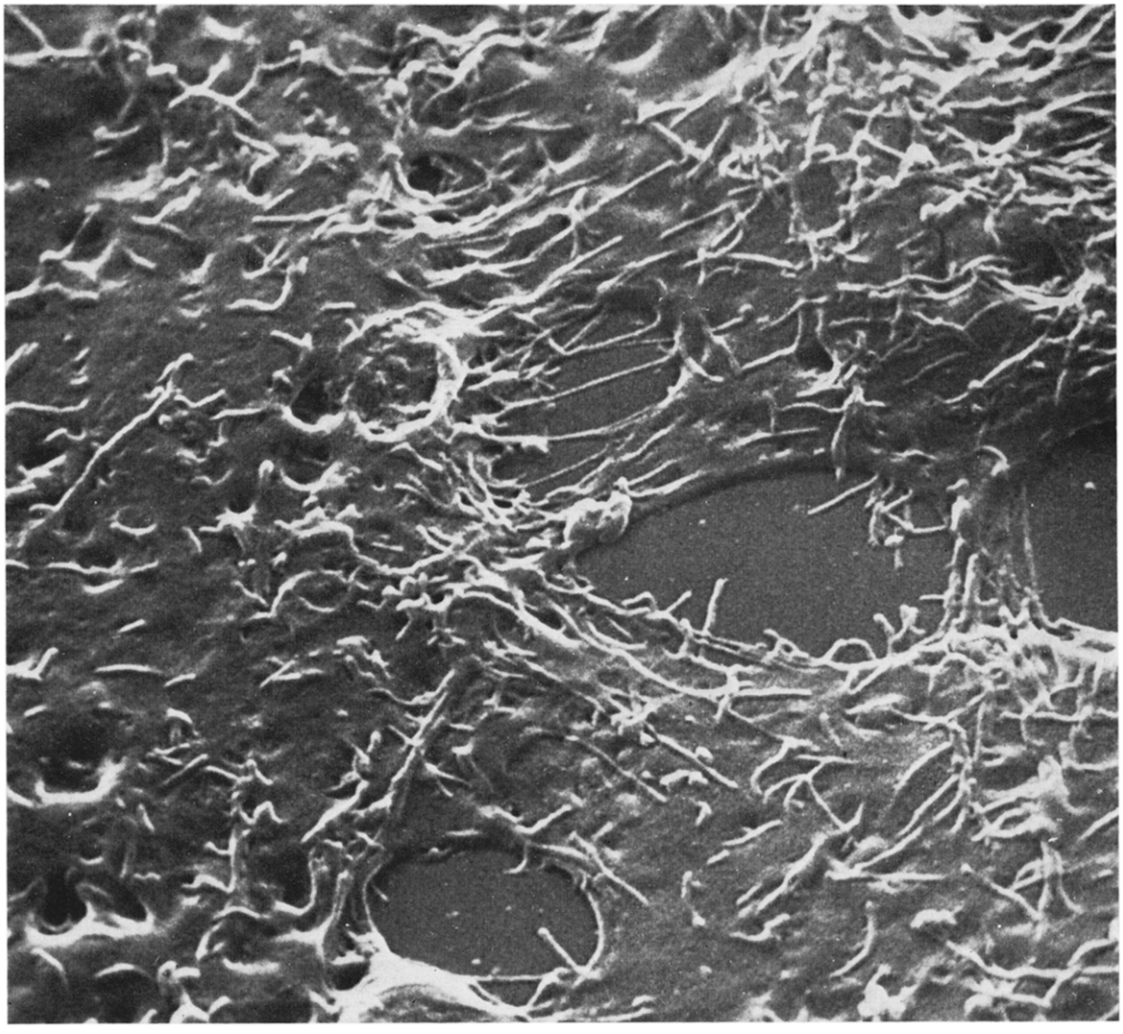

*Fig.* 10. *CMB* 17/42 *culture showing varying complexities of interdigitation between laterally situated microvilli of adjacent cells.* $\times$ 14,400.

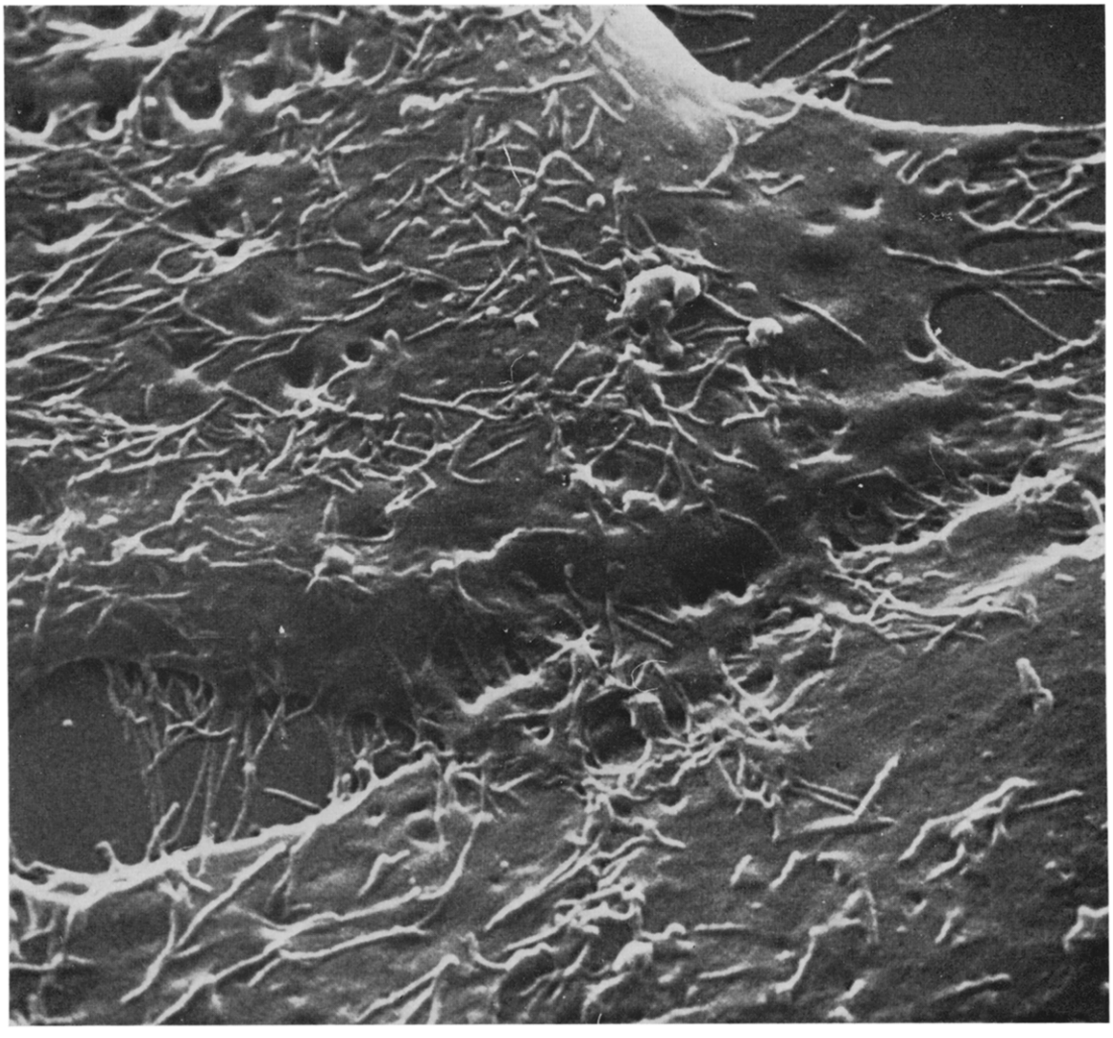

*Fig.* 11. *CBM* 17/42 *culture. Similar to Fig.* 10 *and shows broad lateral connections between cells.* $\times$ 14,400.

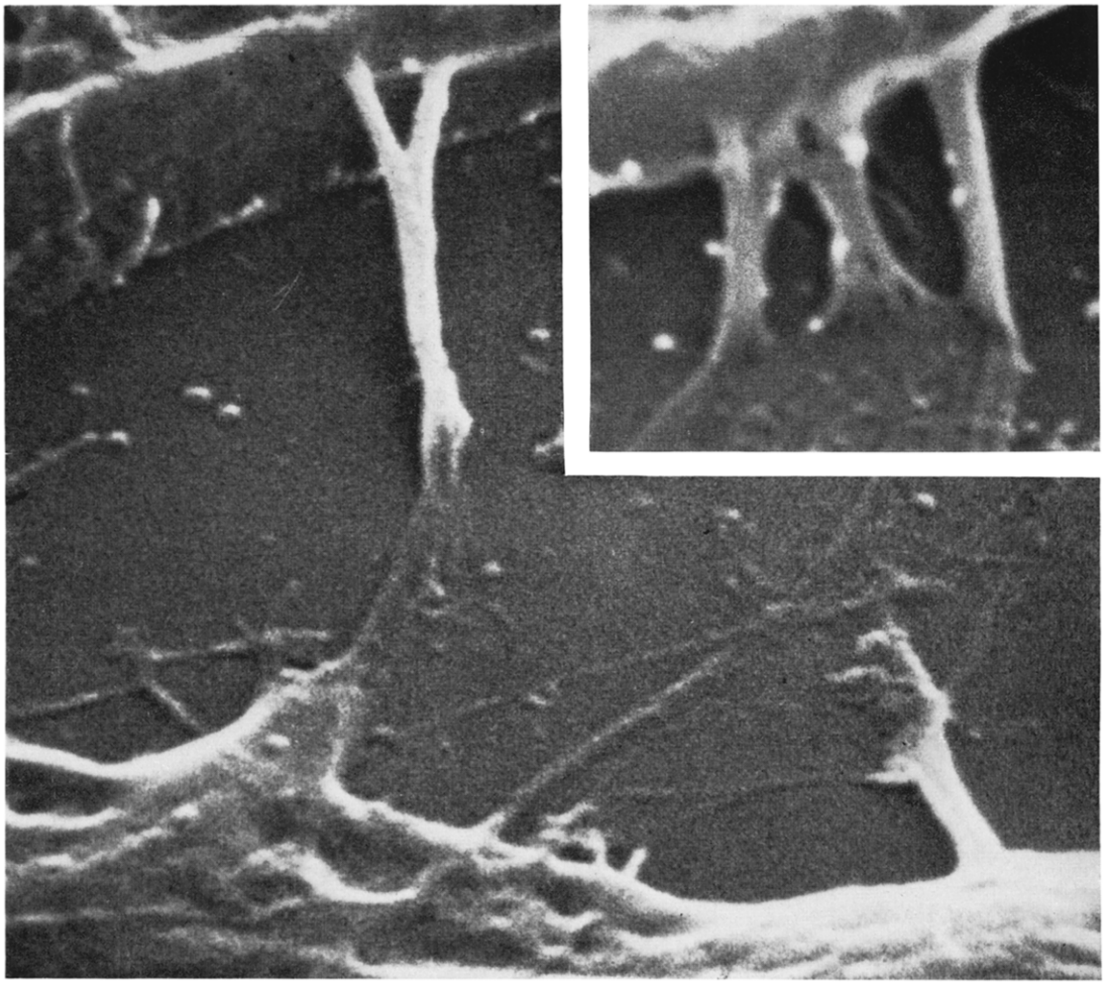

*Fig.* 12. *CBM* 18/37 *culture* (*inset CBM* 17/9 *culture*) *showing adhesion between microvilli and cell membrane of adjoining cells.* ×38,000 (*inset* ×38,000).

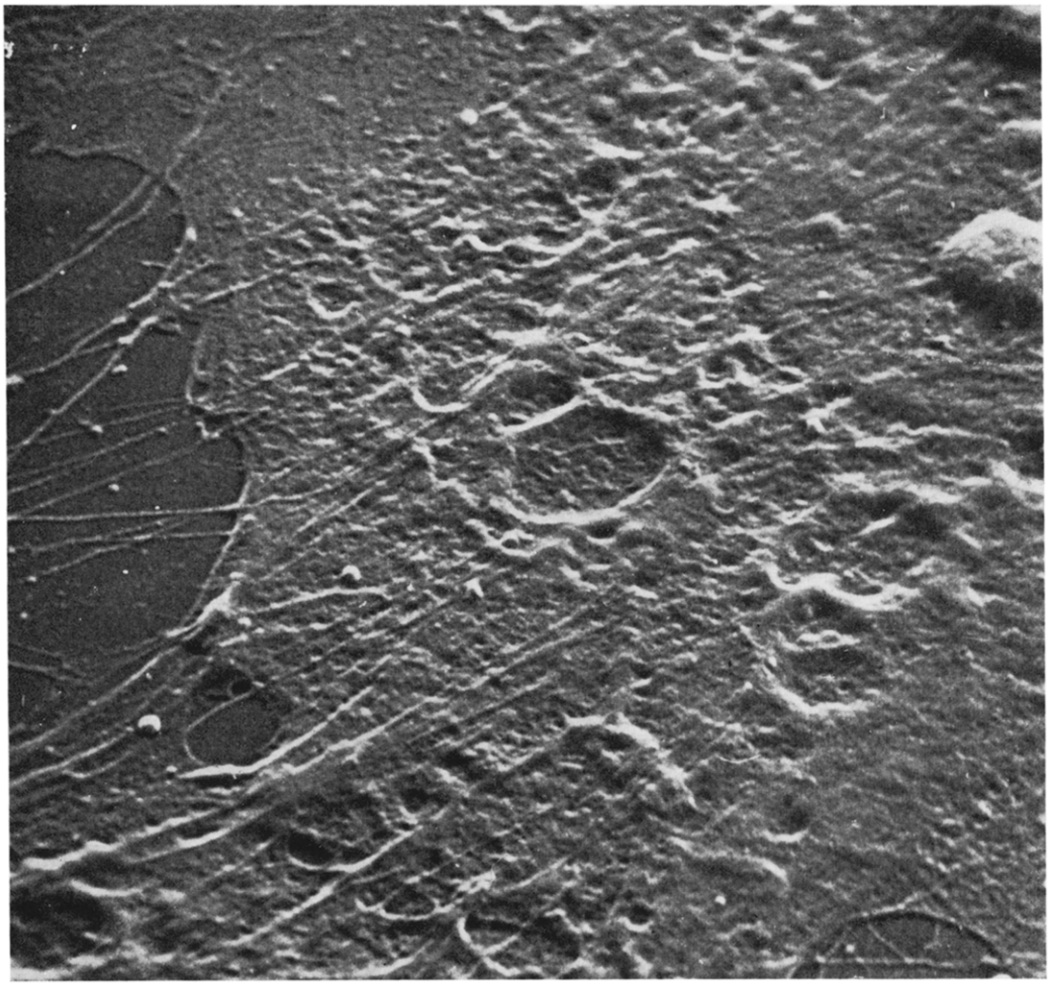

*Fig.* 13. *CBM* 18/37 *culture. Localized junctional region between giant cell and adjoining cell.* × 19,800.

of the polygonal-shaped cells were very similar. Both cell types showed a smooth textured nuclear cytoplasm with prominent protrusions of varying size and shape. The rough-textured cytoplasmic zone was not so clearly defined in either of these cells and merged imperceptibly into a relatively restricted peripheral cytoplasmic region (Figs. 5 and 6). Microvilli were present in variable numbers on most cells and were observed to be in general restricted to the juxtanuclear and peripheral cytoplasm. Some cells, however, had microvilli on the nuclear region and this feature was particularly striking in one culture (CBM 17/42 Fig. 6). The microvilli on the surface of these cells were from 0·06 μ to 1.5 μ in length and 0·03 μ to 0·06 μ in diameter. At the edges of the cells the microvilli were generally characterized as slender, straight or slightly contorted cytoplasmic processes of varying lengths showing occasional bifurcation into unequal branches (Fig. 8). Shorter blunter processes were also encountered showing occasional dilatations which were usually located at the tip. These laterally-situated microvilli varied in length from 1 μ to 4 μ and in diameter from 0·03 μ to 0·18 μ.

A fourth cell type, very similar to the fibroblast-like cell, was occasionally encountered and was characterized by the presence of large open pores or fenestrae in the cytoplasm (Fig. 7).

At higher magnification the surfaces of the different cell types showed a pitted structure but no specific pattern could be distinguished. No distinguishing cell-surface features were seen between the CBM 17 and CBM 18 cultures.

*Cell contacts*

Overlapping between fibroblast-like cells was observed in all cultures (Fig. 5) whilst the polygonal cells formed confluent areas with apposition of lateral surfaces and did not overlap (Fig. 6). Where mixed cell populations occurred the fibroblast-like cells usually overlapped the polygonal cells. There was no evidence of gross overlapping between the giant cell and surrounding cells although occasionally a small portion of the peripheral cytoplasm of these cells did overlap (Figs. 3 and 4). More frequently, the cytoplasmic processes of the fibroblast-like cells crossed over part of the giant cell with the lateral microvilli of the giant cells extending short distances over the adjacent cells.

Attachment of the cells to the underlying glass substratum was by means of numerous fine cytoplasmic extensions the length and diameter of which increased at the cell margin (Fig. 9). Similar attachments were observed in multilayered cell cultures connecting cells to the underlying cell membranes. Portions of the cytoplasmic processes of the cells often remained adherent to the glass (Fig. 9) and could occasionally be seen stretched between two presumably retracting microvilli (Fig. 9).

Laterally-situated microvilli extended for varying distances over neighbouring cells (Fig. 8), those of the fibroblast-like cells being the most extensive. Where apposition of cells occurred the microvilli formed complex junctional networks of interdigitating processes with the adjoining cells (Figs. 6 and 8). Varying complexities of interdigitation could be distinguished depending on the closeness of the two cells to each other (Figs. 6, 10 and 11). Adhesions appeared to form at the pont of contact between individual microvilli of adjacent cells (Fig. 9). Very fine threads of cytoplasm bridging these extensions could be seen where retraction of the microvilli was occurring (Fig. 9) although in general the points of contact were not distinguishable. Occasionally adhesion occurred between microvilli and cell membranes of the laterally adjacent or of the underlying cells (Fig. 12). The lateral connections or cytoplasmic bridges between two cells were generally narrow, where the cells were relatively far apart (Fig. 9) but became progressively broader as more intimate cell–cell contacts were formed (Fig. 11).

The polygonal cells showed lateral attachments which appeared to involve cells of a same morphological type (Fig. 6). The fibroblast-like cells while showing overlapping also revealed close cell–cell adhesions along the lateral edges of the same cell.

In the junctional region between giant cell and surrounding cells few microvilli could be distinguished and direct apposition of surfaces appeared to occur. More intimate cell–cell adhesions were apparent in localised regions of contact (Fig. 13).

## DISCUSSION

The observations indicate that the surface features of the different tissue culture types did not vary significantly from one cell line to another. The surface morphology of CBM 17 remained constant over a prolonged period of time (509 days) although the cell line was tumorigenic by 283 days, at the 23rd culture generation [17].

It is probable that surface topography reflects variations in intracellular dry mass

distribution and is indicative of the presence of underlying structures. Thus the larger protrusions seen in the nuclear cytoplasmic region could correspond to nucleoli while the smaller and more irregular protrusions could represent heterochromatin since both these structures have a greater dry mass than the surrounding nuclear material. The deeper depressions of the nuclear cytoplasm could indicate nuclear pores. It is probable that the rough-textured juxtanuclear cytoplasmic region and polar zone of the cells represent the cisternae of the underlying endoplasmic reticulum and Golgi complex.

Straight striations traversing the surface of the cell are seen in numerous cases and could correspond to bundles of microtubules or to fibrils [18] lying under the cell membrane. The striations have an average width of 1000 Å and this is considerably greater than the 210–230 Å diameter of microtubules reported in transmission electron microscope studies [19]. However, the observed increase in width might be due to the overlying cytoplasm and metallic coating.

Examination of the different cell cultures reveals that the presence and number of microvilli on the upper free surface of the cell is not a constant feature whereas along the lateral margins of the cells there is usually some development of cytoplasmic processes. It has been suggested that the microvilli on the upper free surface of the cell may function to increase the surface area enhancing the transfer of nutrients and synthesized materials [11]. The degree of development of the microvilli is considered to be indicative of the metabolic activity of the cells [20]. The varying development of microvilli on the upper free surface of cells observed in the different cell culture preparations may therefore be morphological expressions of changes in culture conditions and be related to culture age. The presence of microvilli along the lateral edges and on the under surface of the cells suggests that they may be implicated also in the function of cellular attachments and locomotion.

The contact between two cells would appear to start by adhesion of the finer lateral microvilli forming long cytoplasmic connecting "bridges". Communicating intercellular bridges between cells in culture have been reported at the light microscope level using autoradiographic techniques [21, 22] and a similar communicating system may exist in the present cultures. Broader connections are formed as the cells become more closely applied with resultant intimate cell–cell contacts.

The relationship between cells varies according to the cell type involved and the following points can be noted.

1. The polygonal cells form close adhesions along their lateral surfaces with like cells. Overlapping does not occur between like cells but fibroblast cells may overlap the polygonal cells. This suggests that the upper free surface of the polygonal cells exerts a selective inhibitory action on like cells.

2. The fibroblast cells also form lateral adhesions but do not show surface inhibition in that overlapping between like cells and between unlike cells are observed.

3. The giant cells in contrast show surface inhibition and only limited lateral adhesion with the adjoining single cell. The modifications in cell surface properties, as reflected by these local variations in cell–cell relationships, appear related to regional differences in microvilli morphology and possible microvilli function. It is probable that the selective affinities shown by the cultured cells reflect intrinsic cell-type differences. The epithelial cells of the kidney form single-layered membranes with a free upper surface and contacts along the other surfaces. This feature of the simple epithelium would appear to be maintained in culture by the polygonal cells in that their upper surfaces are free and inhibitory to like cells. It would be of interest to know if cells originating from stratified epithelium show any modifications in this cell–cell relationship. In the present scanning electron microscope study different cell types have been recognized based on their morphological features and on differences in their adhesion pattern to adjacent cells. A survey of known cell types *in vitro* is required to define more precisely the intercellular adhesion pattern between like and unlike cells. Modifications in cell–cell relationships during *in vitro* culture and the onset of tumorigenicity in a cell line could as a result be more closely correlated to given cell types.

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## SUMMARY

*The surface appearance and cellular contacts of two mouse kidney cell lines in the process of "spontaneous" transformation have been examined with the Stereoscan scanning electron microscope.*

*The surface features of the four main cell types present is described and correlated to underlying structures. There are no significant differences in the surface topography of the two cell lines. Microvilli show regional differences in development, in morphology and possibly in function. The pattern of apposition between cells varies according to the cell type involved. There are regional differences in cell surface affinities.*


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# Etude Cytologique et Cytochimique des Effets de l'Actinomycine D sur des Fibroblastes de Poulet ou de Rat, Cultivés *in vitro**

ROGER BASSLEER et GUY GOESSENS

*Institut d'Histologie et Centre Anticancéreux, Université de Liège, 20, rue de Pitteurs, Liège (Belgique)*

## INTRODUCTION

On sait que l'actinomycine D, un antibiotique produit par *Streptomyces parvullus* [1], est douée d'une activité cytotoxique et antitumorale [2, 3]. Les nucléoles sont particulièrement sensibles à l'action de l'actinomycine D. En effet, celle-ci provoque, *in vitro* comme *in vivo*, une diminution de la taille et la fragmentation des nucléoles; parfois même ils disparaissent.

Ces faits ont été observés dans des cellules animales très variées [4–11]. Les nucléoles peuvent aussi devenir plus ou moins sphériques [6, 12]. Dans le foie et le pancréas de rats traités par de l'actinomycine D, on note une diminution du volume nucléolaire mais non du volume nucléaire [8]. Sous l'effet de l'actinomycine D, les nucléoles présentent un aspect hétérogène: certaines zones sont intensément colorées par le bleu de toluidine alors que d'autres restent claires. Les zones chromophiles sont localisées à un pôle du nucléole et prennent alors l'aspect de "coiffes" [6, 12, 13], ou forment une couronne périphérique [11, 14, 15]. Certains auteurs ont noté aussi la présence d'"inclusions intranucléaires" contenant notamment de l'acide ribonucléique (ARN) dans des cellules traitées par cet agent cytotoxique [6, 16].

Les modifications ultrastructurales des nucléoles provoquées par l'actinomycine D ont été étudiées par de nombreux auteurs et dans des matériels très variés, [5, 7, 16–20]. Les nucléoles traités présentent un aspect caractéristique. On constate l'apparition de 3 zones: une zone granulaire, une zone fibrillaire et une zone amorphe. Parfois même, une 4ème zone, très dense aux électrons, est visible.

On connaît plusieurs substances capables de provoquer aussi une telle redistribution des composants macromoléculaires du nucléole; ce sont notamment l'aflatoxine, la proflavine et la mitomycine C. Ce phénomène a été appelé "*ségrégation nucléolaire*" par Bernhard *et al.* [21]. Les nucléoles du foie de rats, traités par de l'actinomycine D, présentent cette ségrégation de leurs constituants; après un certain temps, ces nucléoles peuvent cependant retrouver une configuration ultrastructurale normale [22, 23]. Dans le foie de rats ayant reçu de l'actinomycine D après un prétraitement au fluoro-uracile, cette ségrégation ne se produit pas [24]. Simard et Bernhard [25] pensent que les substances agissant directement sur le système de transcription ADN–ARN peuvent produire de la "ségrégation nucléolaire".

L'actinomycine D provoque également l'apparition de grains dans la chromatine des cellules HeLa [13] ou des cellules L de souris [12]. Chez *Artemia salina*, Fautrez-Firlefyn et Fautrez [26] ont observé une coalescence de la chromatine après traitement par de l'actinomycine D.

L'actinomycine D inhibe la multiplication cellulaire et peut provoquer des anomalies de la mitose [27–30].

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Elle provoque l'arrêt des divisions de segmentation chez *Artemia salina* alors que les mitoses de maturation se poursuivent [26]. Par contre, les recherches effectuées chez l'oursin [31] et chez les amphibiens [32] ont montré que l'actinomycine D ne bloque pas les mitoses de segmentation; ses effets n'apparaissent qu'à partir de la gastrulation. Dans des cultures de cellules humaines, Fraccaro *et al.* [10] ont observé que le nombre de mitoses est d'autant plus bas que la concentration d'actinomycine D dans le milieu de culture est élevée. Toutefois, l'actinomycine D ne peut empêcher l'entrée en mitose de cellules traitées à la fin de la période de synthèse des ADN ou en post-synthèse [33, 34]. Goldstein *et al.* [35] ont montré que des cellules HeLa, traitées par de l'actinomycine D (0,1 µg/ml pendant 48 h), peuvent recommencer à se multiplier 5 à 6 jours après lavage et retour en milieu nutritif dépourvu d'actinomycine D. Toutefois selon Clark *et al.* [13], les effets d'un traitement d'une durée de 12 h par de l'actinomycine D, à la concentration de 0,1 µg/ml, sont irréversibles chez les cellules HeLa. Cependant, dans le même matériel, les effets de l'actinomycine D sont réversibles si elle est utilisée à une plus faible concentration (0,01 µg/ml). En effet, lorsque des cultures sont traitées pendant 48 h par cette faible concentration, puis lavées, l'activité mitotique reprend au bout de 2 jours [13].

L'actinomycine D peut provoquer l'apparition de métaphases anormales (fibroblastes de poulet [27]) ou un allongement de la durée de la prophase (foie de souris en régénération [36]). Dans des cellules animales cultivées *in vitro* [10, 37, 38] ou dans des spermatocytes de locuste [39], on observe des cassures de chromatides ou de chromosomes après traitement par de l'actinomycine D.

Les mitochondries de cellules HeLa, traitées par de l'actinomycine D, sont longues et filamenteuses selon Deitch et Godman [40]; par contre, David et Marx [41], et David et Uerlings [42], ont observé un gonflement des mitochondries et des altérations des crêtes mitochondriales dans le foie de rat ou de souris. L'actinomycine D provoque aussi des modifications du réticulum endoplasmique: ce dernier devient vacuolaire [11, 41, 42]. Dans des cellules L de souris traitées par de l'actinomycine D, les polysomes sont dissociés ou peuvent même disparaître. Cet effet est cependant réversible [43].

L'actinomycine D inhibe les synthèses d'ARN. Ce fait a été démontré dans des cellules animales d'origines très variées, *in vivo* ou *in vitro*. La plupart de ces recherches ont été réalisées grâce à la technique histo-autoradiographique ou par des méthodes biochimiques [7, 8, 13, 28, 44–55].

A faible concentration, l'actinomycine D inhibe d'abord les synthèses d'ARN nucléolaire [50, 56, 57]. L'inhibition de l'incorporation des précurseurs d'ARN, provoquée par l'actinomycine D, est cependant réversible dans certaines conditions expérimentales. En effet, Bal et Gross [58], et Mittermayer *et al.* [59], ont montré que l'actinomycine D inhibe l'incorporation d'uracile ou d'uridine tritiées dans des cellules végétales; cependant, lorsque ces cellules sont remises en présence de milieu frais dépourvu d'actinomycine D, l'incorporation de précurseurs tritiés d'ARN reprend et est comparable à celle observée dans les cellules témoins. Dans des amibes traitées par de l'actinomycine D, Lorch et Jeon [60] ont constaté une inhibition de l'incorporation d'uridine tritiée et de la division cellulaire ainsi que des lésions nucléaires. Si le noyau d'une amibe préalablement traitée par de l'actinomycine D est transplanté dans le cytoplasme d'une amibe anucléée mais non traitée, Lorch et Jeon [60] observent une reprise de la division cellulaire et de l'incorporation d'uridine tritiée; de plus, le noyau transplanté retrouve une structure normale.

Dans des cellules animales où les synthèses d'ARN sont inhibées par l'actinomycine D, la duplication de l'acide désoxyribonucléique (ADN) peut se poursuivre [28, 47, 48, 53, 61–65]. Mais au-delà d'une certaine concentration ou après un temps d'action relativement long, l'actinomycine D peut aussi inhiber les synthèses d'ADN [45, 66–69]. Au cours de la période précédant la synthèse des ADN (pré-synthèse ou G1), la cellule serait particulièrement sensible à l'actinomycine D; cette dernière peut en effet empêcher le déclenchement de ces synthèses [12, 63, 64, 69].

Caspersson *et al.* [48] ont montré que, dans des cellules de souris cultivées *in vitro*, l'actinomycine D n'exerce un effet inhibiteur sur les synthèses de protéines qu'à des concentrations relativement élevées. Selon Yaffe et Fuchs [70], à forte concentration, l'actinomycine D est capable d'inhiber l'incoporation de leucine tritiée dans des myoblastes de rat cultivés *in vitro*. Dans des cellules traitées par de l'actinomycine D à faible concentration, les synthèses protéiques peuvent se dérouler de façon quasi normale, alors que les synthèses d'ARN sont inhibées [9, 13, 68, 71].

L'actinomycine D agirait en se fixant sur la molécule d'ADN [45]. Cette fixation entraînerait une inhibition de la synthèse d'ARN dépendant de l'ADN [28, 62].

Goldberg et Rabinowitz [46], Hurwitz *et al.* [47], Reich [72], pensent que l'actinomycine D se lie spécifiquement à la guanine dans le sillon mineur de l'ADN et inhibe l'activité de l'ARN-polymérase; il ne s'agirait donc pas d'un effet direct sur l'enzyme mais bien sur la molécule d'ADN. Ainsi, on est tenté d'admettre que "l'obturation spécifique du sillon mineur de l'ADN hélicoïdal

est le lieu spécifique de la lésion biochimique qui entraîne par la suite une ségrégation, puis l'épuisement du nucléole" [25].

L'utilisation d'actinomycine D, marquée au $^{14}C$ ou au $^{3}H$, a permis de montrer que l'actinomycine D est incorporée dans le noyau de la cellule et se fixe sur les ADN [10, 35, 56, 73–78]. Brachet et Ficq [79, 80], Brachet et Preumont [81] ont mis au point une méthode histo-autoradiographique de détection de l'ADN basée sur l'utilisation d'actinomycine D radioactive. Ces auteurs ont montré que cette méthode est spécifique de l'ADN: elle est applicable à l'étude de cellules fixées et permet la détection de très faibles quantités d'ADN.

Comme nous venons de le voir, de nombreux auteurs ont étudié les effets de l'actinomycine D sur des matériels très variés. Toutefois, très peu de ces travaux ont été réalisés à l'aide de méthodes cytochimiques quantitatives. Dans le cadre de recherches consacrées au mécanisme d'action de substances antimitotiques variées [82], il nous a paru intéressant d'analyser les effets de l'actinomycine D sur les synthèses prémitotiques d'ADN et de protéines, respectivement par cytophotométrie après réaction de Feulgen et par microscopie interférentielle. Nos mesures ont été effectuées dans des fibroblastes et myoblastes de poulet ou de rat cultivés *in vitro*. Ces cellules traitées par de l'actinomycine D ont aussi fait l'objet d'observations en contraste de phase sur le vivant. Une étude histo-autoradiographique des synthèses d'acides nucléiques et de protéines a également été réalisée. Cette dernière méthode nous a permis de détecter en outre dans quelle partie de la cellule l'actinomycine D marquée au tritium est fixée.

Les résultats préliminaires de nos recherches ont été présentés dans les notes suivantes [83–87].

## MATERIEL ET METHODES

1. *Cultures de tissus*

De petits fragments, de 1 $mm^3$ environ, provenant du coeur, du tissu conjonctif sous-cutané ou de muscles squelettiques d'embryons de poulet âgés de 10 à 11 jours ou d'embryons de rat prélevés en fin de gestation ont été mis en culture sur doubles lamelles et lames de Maximow. Les cellules de poulet ont été cultivées en caillot ou en milieu liquide. Le caillot contenait 1 goutte de plasma de coq au 1/8, 1 goutte de sérum de coq au 1/3, 1 goutte d'extrait embryonnaire de poulet au 1/3 et une goutte de liquide de Tyrode-glycosol. Ce caillot a été ensuite aminci. Dans le cas des cultures en milieu liquide, le milieu nutritif se composait de 3 parties de sérum de coq, de 6 parties de milieu de culture NCTC 109 Difco et de 1 partie de liquide de Tyrode-glycosol. Les fibroblastes de rat ont été cultivés en milieu liquide; ce dernier se composait de 3 parties de sérum de veau, 4 parties de milieu de culture NCTC 109 Difco et de 3 parties de liquide de Tyrode-glycosol. Tous les 2 ou 3 jours, les cultures en caillot ou en milieu liquide ont été "entretenues", c'est-à-dire aérées, lavées au liquide de Tyrode-glycosol à 37°C, et le milieu nutritif a été renouvelé sans repiquage des explants. Dans ces conditions, la multiplication cellulaire est très active. Autour des explants, on observe surtout des fibroblastes, quelques histiocytes et macrophages ainsi que des myoblastes. Ces derniers prennent un aspect fibroblastique. Dans le présent travail, fibroblastes et myoblastes seront appelés "fibroblastes".

De l'actinomycine D de marque "Merk, Sharp and Dohme" a été ajoutée au milieu nutritif de cellules mises en culture depuis 2 à 3 jours, à des concentrations finales variant entre 1 µg/ml et 0,005 µg/ml. La durée du traitement a été en général de quelques minutes à 2–3 jours, mais, pour une faible concentration d'actinomycine D (0,025 µg/ml), le traitement a duré jusqu'à 8 jours.

2. *Observation des cellules sur le vivant*

Les lames de culture non démontées ont été examinées pendant de courtes périodes (quelques minutes) à l'aide d'un microscope équipé du dispositif interférentiel selon Nomarski; rappelons que ce système permet d'obtenir des images avec une impression de relief. En vue de réaliser des observations de longue durée (de quelques heures à 2 jours), les cellules ont été cultivées en caillot. La lamelle portant les cultures a été scellée sur une lame creuse spéciale [88]. Ces cultures ont été observées sur le vivant, à 37°C, à l'aide d'un microscope à contraste de phase équipé d'une platine chauffante.

3. *Fixations et colorations*

Les cultures ont été lavées au liquide de Tyrode-glycosol à 37°C, puis fixées soit aux vapeurs d'acide osmique puis à l'alcool éthylique à 80 vol.%, soit dans un mélange à parties égales d'éthanol absolu et d'acétone, à 4°C, pendant 24 h. Cette dernière méthode de fixation s'est révélée excellente, en particulier pour les dosages micro-interférentiels.

Des cultures ont été colorées par l'hématoxyline ferrique selon von Möllendorff ou par le bleu de toluidine.

4. *Index mitotiques*

Les index mitotiques ont été établis en observant, dans chaque cas, 3.000 à 7.000 cellules; ils ont été exprimés en nombres moyens de mitoses pour 1.000 cellules.

5. *Dosages cytophotometriques d'ADN.*

La teneur en ADN de noyaux en interphase et de figures mitotiques a été déterminée par cytophotométrie après réaction de Feulgen et en lumière visible dans des cellules considérées individuellement. Pour réaliser cette réaction, l'hydrolyse a été du type classique (HCL N à 56°C, pendant 20 min., temps optimal pour notre

matériel [82]. Des dosages cytophotométriques d'ADN ont été effectués en lumière monochromatique (543 mμ) à l'aide du microdensitomètre intégrateur de Barr and Stroud muni d'un système "scanning" selon Deeley [89]. La teneur en ADN ainsi obtenue a été exprimée en unités arbitraires. Tous nos dosages ont été réalisés dans la zone de croissance des cultures en parcourant les rayons de cette zone mais sans opérer de choix parmi les cellules recontrées. Cependant, nous avons aussi choisi, puis mesuré, des figures mitotiques et des noyaux présentant un aspect granulo-filamenteux (voir plus loin). Pour chaque culture étudiée, 50 cellules au moins ont été mesurées et l'index mitotique a été établi.

6. *Mesure du poids sec total de noyaux et de nucléoles par microscopie interférentielle*

Des mesures de poids sec nucléaire total (nucléoles non compris) ont été réalisées, après fixation à l'éthanol absolu - acétone, dans des fibroblastes de rat considérés individuellement, à l'aide du microscope à interférence de Smith-Baker et de son oculaire spécial "half-shade" [90]. Une description détaillée de cette méthode a été présentée récemment [82]. Après repérage des cellules ainsi étudiées, la réaction de Feulgen a été réalisée. La teneur en ADN des noyaux qui avaient été préalablement mesurés par microscopie à interférence a été ensuite déterminée par cytophotométrie. La teneur en protéines nucléaires totales a été calculée en admettant que celle-ci correspond au résultat de la soustraction: poids sec total du noyau moins la teneur en ADN correspondante. Les résultats des dosages cytophotométriques d'ADN ont donc dû être préalablement transformés en valeurs absolues; la valeur diploïde moyenne pour le rat, trouvée par méthode biochimique et utilisée ici, est de $5{,}7 \times 10^{-12}$ g [91]. Le poids sec total du matériel correspondant à l'aire de projection des nucléoles a été aussi déterminé par la méthode interférentielle; lorsque le noyau contenait plusieurs nucléoles, les poids secs des différents nucléoles ont été additionnés donnant ainsi, pour le matériel nucléolaire, une valeur totale par noyau.

7. *Etude histo-autoradiographique*

Nous avons étudié, dans des fibroblastes de rat, le métabolisme et la synthèse des acides nucléiques et des protéines à l'aide de la technique histo-autoradiographique basée sur l'incorporation de précurseurs tritiés (de marque "Amersham"). De la thymidine (activité spécifique: 3Ci/mM), de l'uridine (activité spécifique: 3,33Ci/mM) ou de la leucine (activité spécifique: 0,2 Ci/mM) a été mélangée au milieu nutritif de cellules mises en cultures depuis 72 h (concentration finale: 10 μCi/ml). De l'actinomycine D (concentration finale: 0,5 μg/ml) a été ajoutée au même moment que le précurseur. Après 24 h de contact avec le précurseur et l'antimitotique, les cultures ont été lavées au liquide de Tyrode-glycosol à 37°C puis fixées aux vapeurs d'acide osmique et à l'alcool éthylique à 80 vol.%. Dans d'autres cas, nous avons ajouté de la thymidine (activité spécifique: 2 Ci/mM), de l'uridine (activité spécifique: 2,4 Ci/mM) ou de la leucine (activité spécifique: 0,2 Ci/mM) tritiées (concentration finale: 10 μCi/ml) au milieu nutritif de cellules qui avaient été préalablement traitées par de l'actinomycine D pendant 24 h à la concentration finale de 0,5 μg/ml ou pendant 72 h à la concentration finale de 0,025 μg/ml. Après un séjour de 3 h 30 dans le milieu nutritif contenant le précurseur et l'actinomycine D, les cultures ont été lavées puis fixées aux vapeurs d'acide osmique et à l'alcool éthylique à 80 vol.%. Notons que, dans le cas de l'uridine tritiée, les cultures fixées ont été traitées par une solution de désoxyribonucléase neutre de marque "Worthington" (DNase neutre à 1/20.000, pendant 24 h, à 37°C, en présence de $MgCl_2$ à 0,2 M) en vue d'éliminer le marquage cellulaire au niveau de l'ADN [92]. Notre technique histo-autoradiographique est celle du "stripping-film" telle qu'elle a été décrite par Chèvremont *et al.* [93]. Le temps de pose a été de 8 jours à 4°C. Les cellules ont été colorées par l'hématoxyline d'Ehrlich.

A l'aide d'actinomycine D tritiée, nous avons également étudié la localisation de cet antimitotique dans notre matériel. De l'actinomycine D tritiée de marque "Schwarz" (activité spécifique: 3,38 Ci/mM; concentration finale: 5 μCi/ml) a été mélangée au milieu nutritif de cellules mises en culture depuis 48 h., et non traitées. Après 2 h. de contact, les cultures ont été lavées, puis fixées aux vapeurs d'acide osmique et à l'alcool éthylique à 80 vol.%. Nous avons également utilisé la technique dite de "coloration" par l'actinomycine D radioactive, mise au point par Brachet et Ficq [79, 80], Brachet et Preumont [81]. Des cultures âgées de 48 h., et non traitées, one été lavées puis fixées aux vapeurs d'acide osmique et à l'alcool éthylique à 80 vol.%. Ces cultures fixées ont été recouvertes par une solution d'actinomycine D tritiée (concentration finale: 5 μCi/ml) pendant 2 h. Les cultures ont ensuite été rincées dans une solution d'actinomycine D non radioactive, puis lavées pendant 12 h. à l'eau courante. Certaines cultures traitées par l'actinomycine D tritiée sur le vivant, ou après fixation, one subi une digestion par la DNase neutre (voir plus haut) après la fixation histologique en vue de vérifier si l'actinomycine D se lie exclusivement et de manière spécifique aux ADN comme d'autres auteurs l'avaient montré pour d'autres matériels. Les cultures traitées par de l'actinomycine D tritiée ont été étudiées par la technique histo-autoradiographique décrite ci-dessus. Le temps de pose a été ici de 6 semaines.

## RESULTATS PERSONNELS

*A. Etude cytologique de fibroblastes traités vivants par de l'actinomycine D.*

1. *Examens en contraste de phase sur le vivant.*— Des fibroblastes de poulet ou de rat ont été

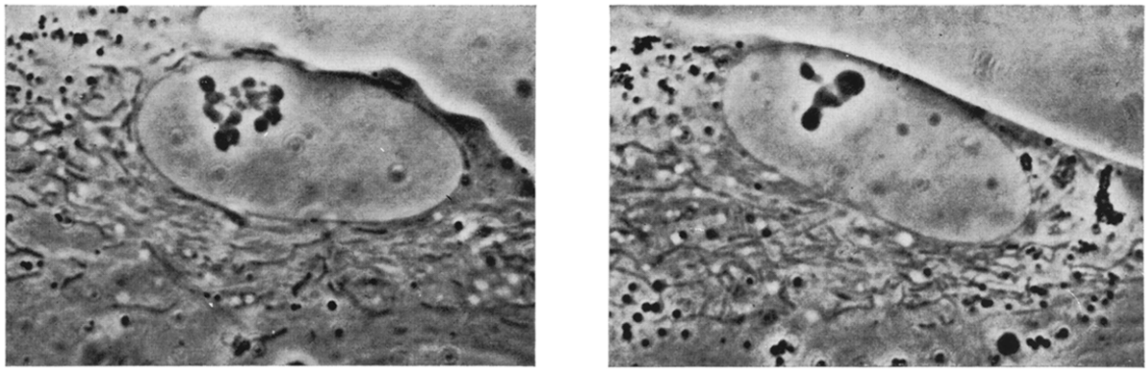

*Fig.* 1. *Fibroblaste de poulet traité par de l'actinomycine D et examiné en contraste de phase sur le vivant. A gauche: aspect de la cellule au début du traitement* (0·5 μg/*ml*). *A droite: même cellule après* 9 *h de traitement; le nucléole est nettement plus petit et se fragmente. Les mitochondries restent filamenteuses et mobiles* (*Agr.* × 3150).

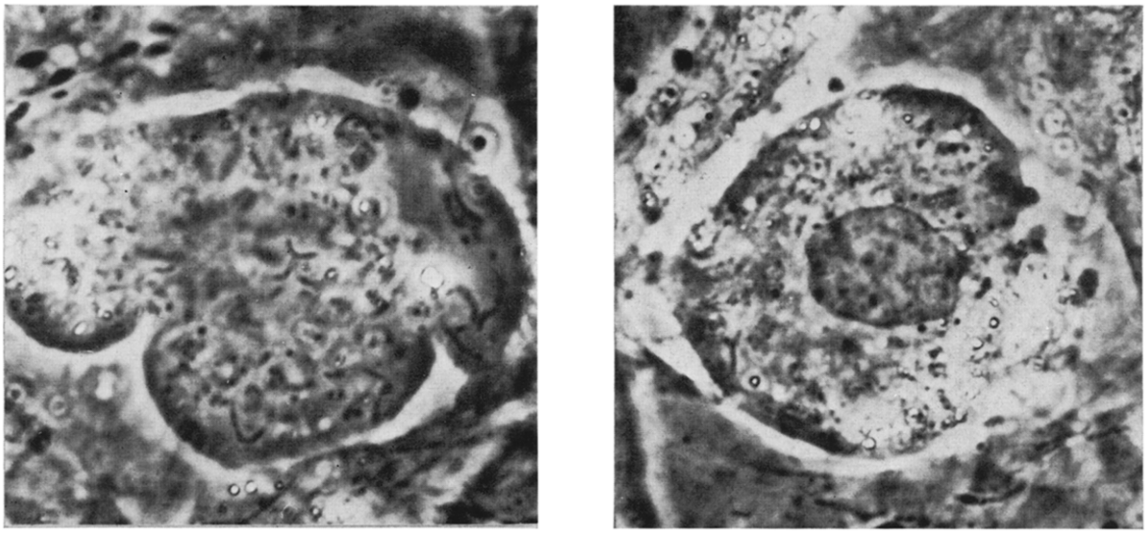

*Fig.* 2. *Perturbation de la mitose provoquée par l'actinomycine D dans un fibroblaste de poulet. La cellule est examinée vivante, en contraste de phase. A gauche: la cellule est en métaphase,* 2 *h après le début du traitement* (0·5 μg/*ml*). *Cette métaphase est restée bloquée pendant* 15 *h* 30. *A droite:* 20 h 15 *après le début du traitement, un seul noyau s'est reconstitué dans la cellule qui n'a pas réussi à se diviser* (*Agr.* × 1750).

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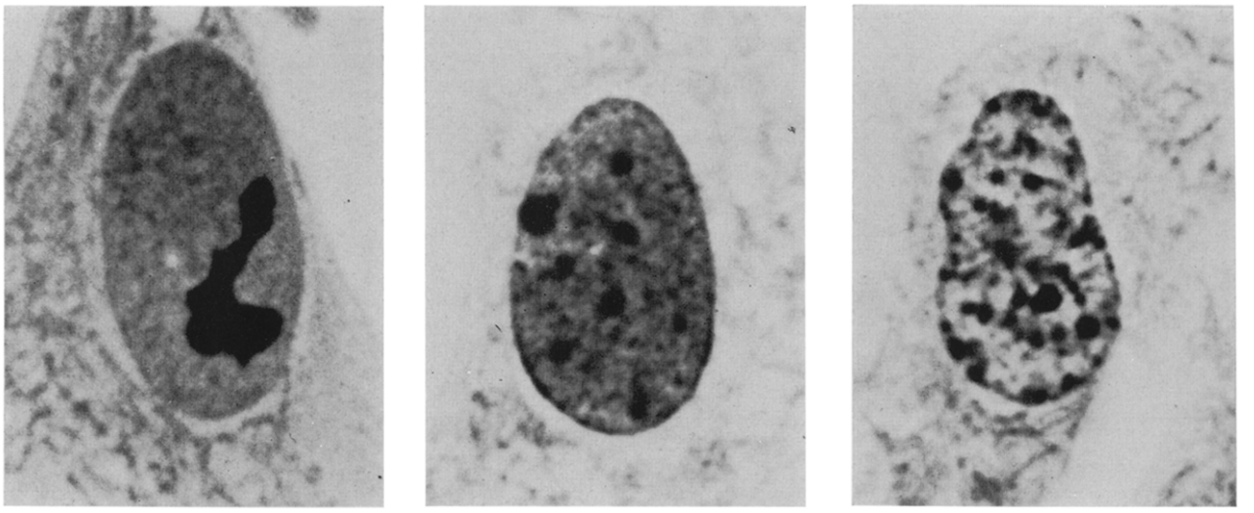

*Fig.* 3. *Altérations nucléaires et nucléolaires provoquées par l'actinomycine D dans des fibroblastes de poulet. Les cellules ont été fixées, puis colorées par l'hématoxyline ferrique selon von Möllendorff. A gauche: fibroblaste témoin; remarquer le volumineux nucléole. Au milieu et à droite: fibroblastes traités vivants pendant* 6 *h par de l'actinomycine D* (0·5 μg/*ml*). *Au milieu: fragmentation nucléolaire. A droite: aspect granulo-filamenteux* (*pseudo-prophasique*) *du noyau* (*Agr.* × 3000).

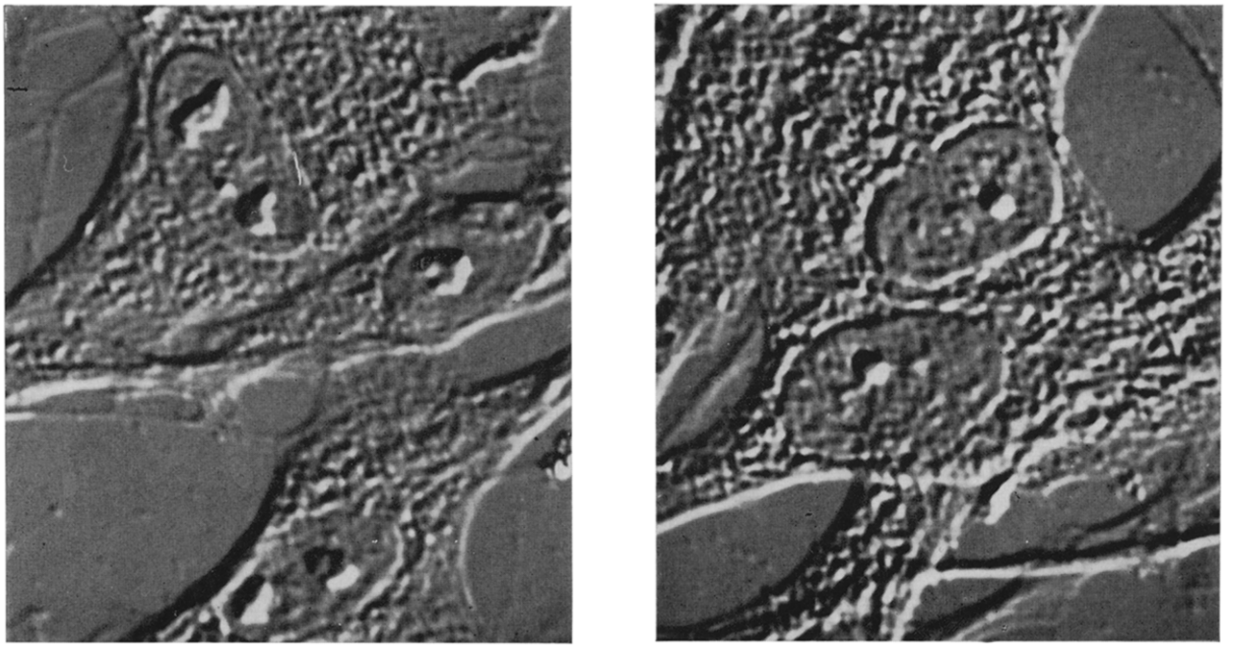

*Fig.* 4. *Effet de l'actinomycine D sur les nucléoles dans des fibroblastes de poulet. Les cellules ont été fixées et sont examinées en contraste interférentiel selon Nomarski. A gauche: fibroblastes témoins. A droite: fibroblastes traités pendant* 21 *h* 30 *par de l'actinomycine D* (0·5 μg/*ml*). *Remarquer la forte diminution de la taille des nucléoles qui tendent à s'arrondir* (*Agr.* × 1400).

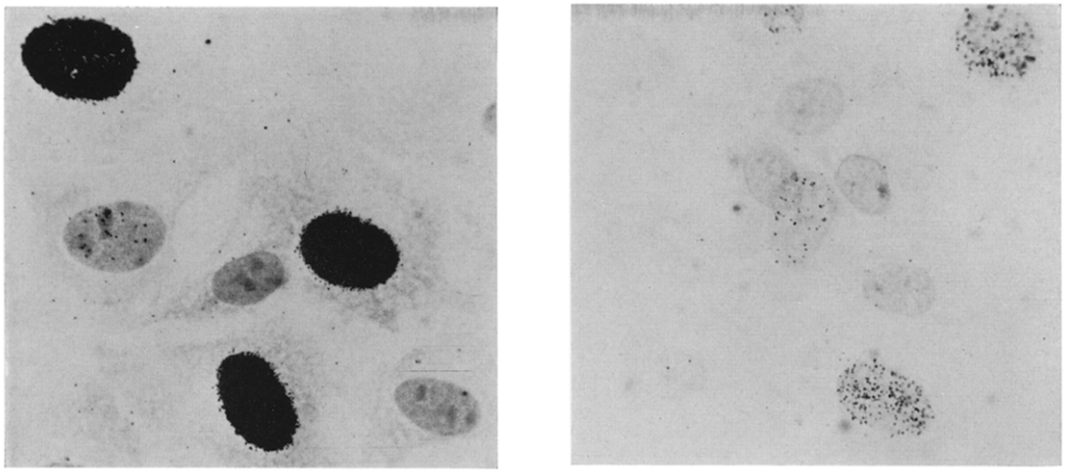

*Fig.* 8. *Incorporation de thymidine tritiée dans des fibroblastes de rat traités par de l'actinomycine D et dans les témoins correspondants. A gauche: cellules témoins. A droite: cellules traitées pendant* 27 *h* 30 (0·5 μg/*ml*). *Comme pour les figures* 9 *et* 10 *suivantes: cultures mises au contact du précurseur radioactif pendant* 3 *h* 30, *histo-autoradiographies réalisées par la technique du stripping-film, coloration par l'hématoxyline d'Ehrlich* (*Agr.* ×625).

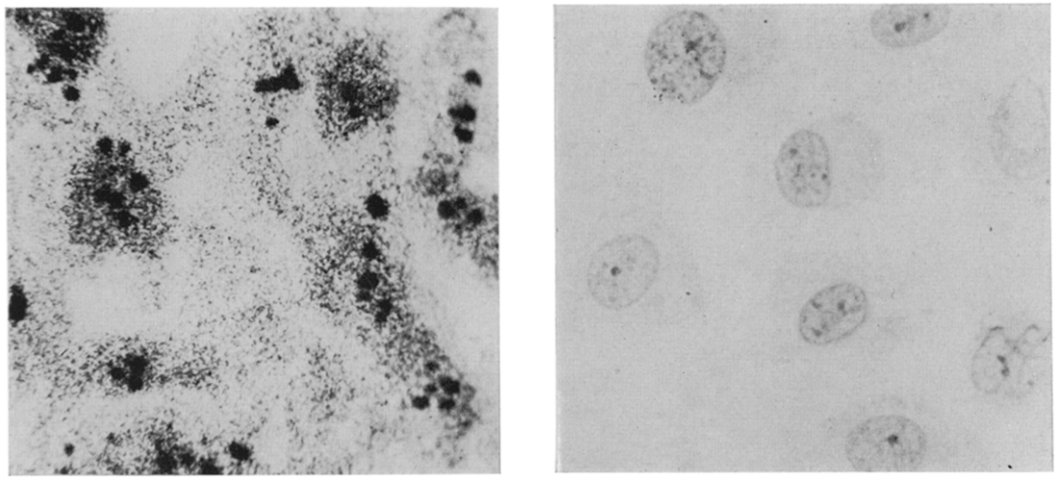

*Fig.* 9. *Incorporation d'uridine tritiée dans des fibroblastes de rat traités par de l'actinomycine D et dans les témoins correspondants. A gauche: cellules témoins. A droite: cellules traitées pendant* 27 *h* 30 (0·5 μg/*ml*).

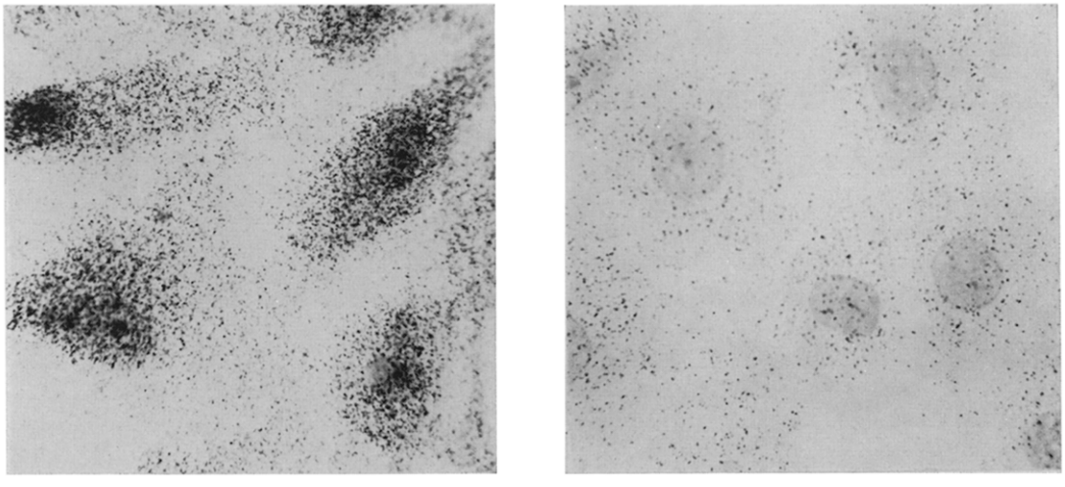

*Fig.* 10. *Incorporation de leucine tritiée dans des fibroblastes de rat traités par de l'actinomycine D et dans les témoins correspondants. A gauche: cellules témoins. A droite: cellules traitées pendant* 27 *h* 30 (0·5 μg/*ml*).

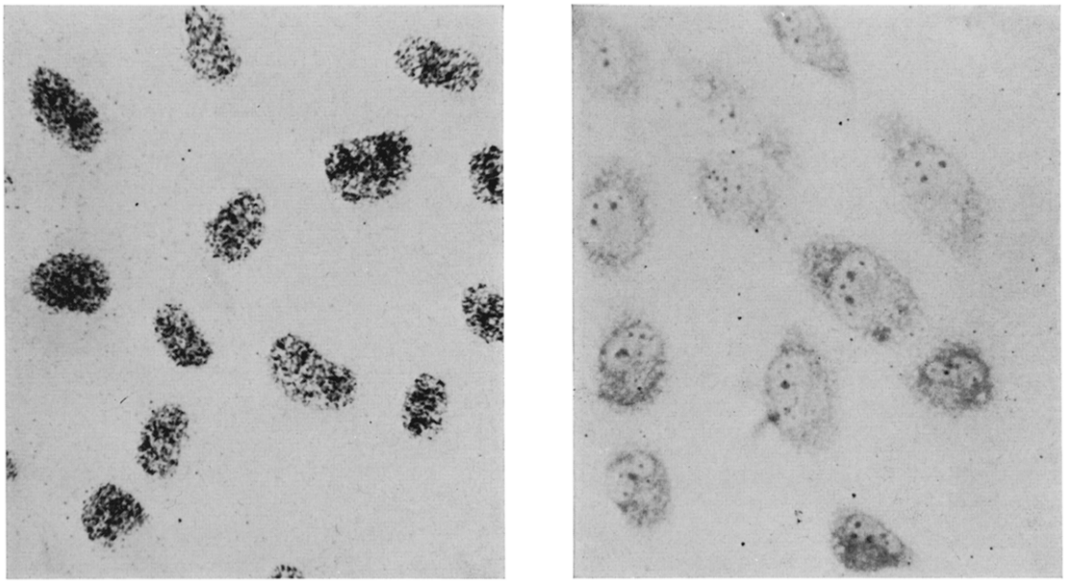

*Fig.* 11. *Fixation de l'actinomycine D tritiée au niveau du noyau dans des fibroblastes de rat. Des fibroblastes non traités ont été préalablement fixés puis mis au contact d'actinomycine D tritiée. A gauche: le noyau est bien marqué et le cytoplasme ne l'est pas. A droite: des fibroblastes ont été fixés, mis au contact d'actinomycine D tritiée puis ont subi une digestion par de la désoxyribonucléase neutre. On remarque que, dans ces conditions, le marquage par l'actinomycine D tritiée a disparu. Histo-autoradiographies réalisées par la technique du stripping-film, coloration par l'hématoxyline d'Ehrlich* (*Agr.* $\times 625$).

cultivés pendant 48 ou 72 h, puis placés dans un milieu nutritif contenant 0,5 µg d'actinomycine D par ml. Ces cellules ont été examinées en contraste de phase et à 37°C. Dans les cellules en interphase, les modifications des nucléoles sont rapides. Dès les premières heures, la taille de ces derniers diminue et ils deviennent très denses; souvent, ils se fragmentent (Fig. 1). Après 2 à 3 h de traitement, quelques noyaux sont granulo-filamenteux. Quant aux mitochondries, leur aspect est le plus souvent normal; leurs mouvements sont très actifs et elles restent filamenteuses (Fig. 1).

La durée de la mitose est souvent allongée. La métaphase peut durer jusqu'à 21 h. Habituellement, la division se termine cependant de façon normale, mais, dans certains cas, une seule cellule binucléée ou à noyau unique (Fig. 2) se reconstitue.

2. *Aspect des cellules après fixation.*—Dans les fibroblastes de poulet ou de rat traités par de l'actinomycine D (0,005 µg/ml à 1 µg/ml), les modifications cytologiques sont les suivantes. Les nucléoles sont très petits, denses, arrondis et souvent fragmentés (Fig. 3 et 4). Après coloration au bleu de toluidine, on distingue parfois deux zones dans le nucléole: une zone basophile bien colorée et une zone incolore ou très peu colorable. Il est possible que ce fait soit en relation avec la "ségrégation nucléolaire" (voir Introduction). Après réaction de Feulgen, la chromatine périnucléolaire est colorée et entoure le nucléole incolore; elle prend souvent l'aspect d'une "coiffe".

Quelques noyaux sont granulo-filamenteux (Fig. 3); ces grains et ces filaments sont basophiles et le Feulgen positif. Par la suite, ces noyaux subissent une dégénérescence du type caryorrhexis. Dans les figures mitotiques, nous n'avons observé ni chromosomes aberrants ni cassures de chromosomes.

En ce qui concerne le cytoplasme, on note une diminution de la basophilie, déjà après 6 heures de traitement.

3. *Activité mitotique.*—Dans toutes nos conditions expérimentales, même après un traitement par une très faible concentration (0,005 µg/ml), les index mitotiques sont très faibles (Fig. 5 et 6). La chute de l'activité mitotique s'observe parfois dès la 2e h du traitement et se maintient pendant toute la durée de l'expérience. Très souvent, les index mitotiques sont inférieurs à 1%. On note une légère augmentation des pourcentages de métaphases; la durée de celles-ci est en effet souvent allongée.

*B. Effets de l'actinomycine D sur les synthèses d'acides nucléiques et de protéines*

1. *Dosages cytophotométriques d'ADN après réaction de Feulgen.*—D'une manière générale, nos dosages d'ADN dans des fibroblastes traités par de l'actinomycine D ont montré que la teneur diploïde moyenne en ADN n'est

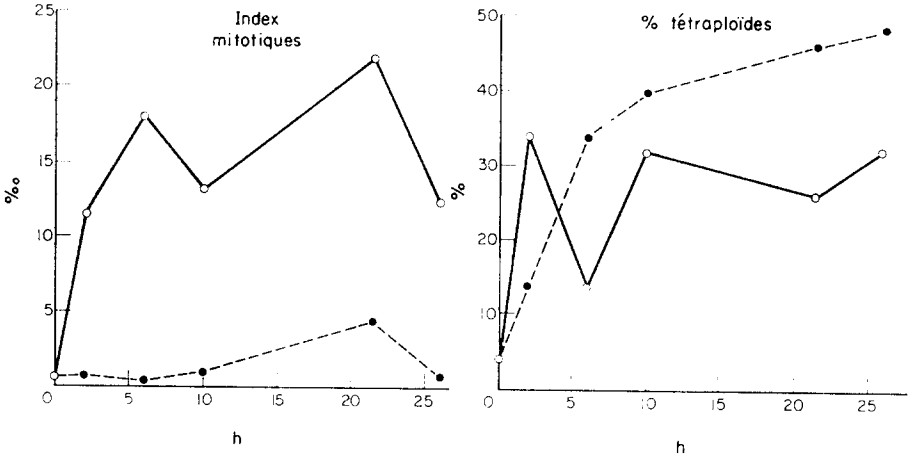


*Fig. 5. Activité mitotique et pourcentages de noyaux tétraploïdes quant aux ADN dans des cultures de fibroblastes de rat traitées par de l'actinomycine D et dans les témoins correspondants. A gauche: les index mitotiques (nombres moyens de mitoses pour* 1000 *cellules) sont portés en ordonnées. En abscisses, le temps exprimé en heures. Comme pour le diagramme situé dans la partie droite de cette figure, les cercles clairs et les cercles noirs correspondent aux valeurs trouvées respectivement chez les témoins et les traités. Entre les cercles clairs ou les cercles noirs, des lignes droites continues ou interrompues ont été tracées pour une raison purement graphique, afin de rendre mieux lisible cette figure. A droite: les pourcentages de noyaux tétraploïdes (4 ADN), observés dans des cultures ou les index mitotiques, ont été établis sont portés en ordonnées; en abscisses, le temps exprimé en heures. L'actinomycine D (concentration finale:* 0·5 µg/ml) *est présente dans le milieu de culture pendant toute la durée de l'expérience* (26 *h*).

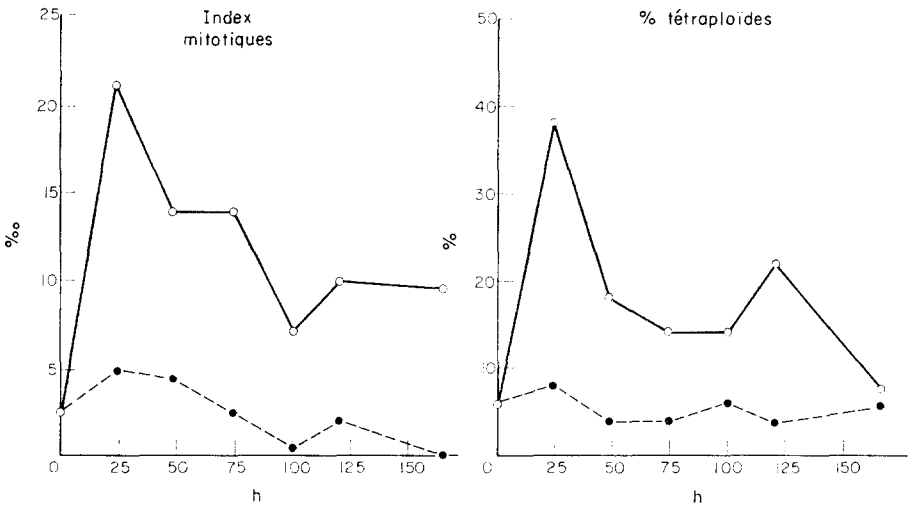


*Fig. 6. Activité mitotique et pourcentages de noyaux tétraploïdes quant aux ADN dans des cultures de fibroblastes de rat traitées par l'actinomycine D et dans les témoins correspondants. La construction de ces diagrammes est identique à celle de la figure 5. L'actinomycine D (concentration finale de 0·025 µg/ml) est présente dans le milieu de culture pendant toute la durée de l'expérience. Ici, la durée du traitement est relativement longue (165 h). Les cultures ont été entretenues à 0 h, à 48 h et à 96 h.*

pas modifiée, du moins dans les limites de précision de nos mesures. Les mitoses sont normales en ce qui concerne la teneur en ADN (valeurs tétraploïdes ou 4 ADN). Il ne se forme pas de cellules polyploïdes quant aux ADN (valeurs supérieures à 4 ADN) sous l'effet du traitement.

Dans la Fig. 5, nous avons rassemblé les résultats obtenus pour des fibroblastes de rat traités pendant 26 h par de l'actinomycine D (0,5 µg/ml). Les cellules sont d'abord mises en culture pendant 72 h; ensuite, elles sont "entretenues", c'est-à-dire lavées, aérées et replacées dans du milieu nutritif frais. Vingt-quatre heures plus tard, l'expérience commence: les cultures subissent un nouvel entretien et certaines d'entre elles reçoivent de l'actinomycine D. Suite à ces deux entretiens successifs, l'activité mitotique augmente fortement chez les témoins et se maintient à un niveau relativement élevé (Fig. 5). Sous l'effet du traitement, les figures mitotiques restent rares. Les pourcentages de cellules prémitotiques ou tétraploïdes quant aux ADN deviennent rapidement plus élevés que chez les témoins (Fig. 5). Chez ces derniers, la diminution du pourcentage de cellules tétraploïdes observée à la 6e h s'explique par le déclenchement de nombreuses mitoses à ce moment. Par la suite, les pourcentages de cellules à 4 ADN continuent de s'accroître chez les traités, mais à un rythme moins élevé qu'en début d'expérience. Comme les index mitotiques sont très bas depuis le début du traitement, nous en concluons que l'actinomycine D inhibe l'entrée en mitose; cependant, beaucoup de cellules atteignent la préprophase. L'accumulation de noyaux tétraploïdes est moins forte vers la fin de l'expérience parce que les synthèses d'ADN subissent une certaine inhibition. C'est aussi la raison pour laquelle un pourcentage élevé de cellules restent diploïdes quant aux ADN. En ce qui concerne les noyaux granulo-filamenteux décrits plus haut, ils sont soit diploïdes, soit tétraploïdes quant aux ADN. Il s'agit donc bien d'une dégénérescence progressive du noyau qui n'est pas nécessairement liée à un blocage en préprophase.

Le même type d'expérience a été réalisé en faisant agir sur des fibroblastes de rat, de l'actinomycine D à une concentration plus faible (0,025 µg/ml) mais pendant 165 h (Fig. 6). Ici, le traitement a été appliqué à des cultures âgées de 72 h, non encore entretenues, où l'index mitotique n'était que de 2,5°/°°. L'entretien des cultures et l'addition d'actinomycine au milieu nutritif de certaines d'entre elles ont eu lieu à 0 h, 48 h et 96 h, (Fig. 6). Suite au premier entretien, l'activité mitotique reprend nettement chez les témoins. Chez les traités, par contre, les index mitotiques restent faibles pendant toute l'expérience. Cet entretien a aussi pour conséquence de provoquer une augmentation des pourcentages de cellules tétraploïdes quant aux ADN chez les témoins (Fig. 6). Cependant, les pourcentages de cellules à 4 ADN restent faibles chez les traités jusqu'à la fin de l'expérience. Nous constatons donc que l'effet inhibiteur de l'actinomycine D sur les synthèses d'ADN est plus intense ici que dans l'expérience précédente (Fig. 5) bien que la concentration utilisée soit plus faible.

Nous pensons que cela peut s'expliquer de la façon suivante: le traitement a débuté alors que les cultures étaient en fin de croissance et de ce fait peut-être plus sensibles à l'actinomycine.

Les pourcentages de cellules tétraploïdes (4 ADN) observés dans les cultures traitées résultent donc, d'une part, d'une certaine accumulation de noyaux tétraploïdes due au blocage de l'entrée en mitose, mais l'inhibition des synthèses d'ADN, d'autre part, tend à diminuer le pourcentage de ces cellules tétraploïdes. Suivant les circonstances, l'un ou l'autre de ces effets prédomine.

Des fibroblastes de poulet ont été traités par une forte concentration d'actinomycine D (1 µg/ml) pendant 7 h. Les pourcentages de cellules tétraploïdes (4 ADN) ne sont que de 6 contre 26 chez les témoins; l'index mitotique est de 0 °/°° dans ces cultures traitées et de 4 °/°° chez les témoins. Les noyaux granulo-filamenteux sont nombreux et leur teneur en ADN est soit diploïde soit tétraploïde, comme chez le rat. A ces divers points de vue, les effets de l'actinomycine D sont donc semblables dans les cellules de Mammifère ou d'Oiseau.

2. *Dosages micro-interférentiels de la teneur en protéines nucléaires totales et de la teneur en matière sèche nucléolaire.*—Dans une première expérience, des fibroblastes de rat ont été traités pendant 25 h par de l'actinomycine D à la concentration finale de 0.5 µg/ml. La teneur en protéines totales du noyau et la teneur en ADN ont été déterminées successivement dans les mêmes cellules traitées.

Les nucléoles sont devenus petits et très denses; l'activité mitotique n'est que de 1,5 °/°° contre 13 °/°° dans les cultures témoins. Dans la figure 7, nous avons étudié la relation entre la teneur en protéines nucléaires totales et la teneur en ADN; pour les cellules témoins, le diagramme présente l'allure habituelle [82]. Les cellules en début d'interphase (ou diploïdes) se caractérisent par une teneur en protéines nucléaires totales (2 p) de 32,5 pg en moyenne et une teneur en ADN (2 ADN) de 5,7 pg. Quelques cellules sont en train de réaliser des synthèses prémitotiques de protéines nucléaires et d'ADN. Quant aux points situés dans la partie droite de ce diagramme, la plupart correspondent à des cellules en préprophase, tétraploïdes quant aux protéines nucléaires et quant aux ADN (4p/4ADN). Dans cette culture témoin, environ 26% des cellules ont réalisé la duplication de leur matériel nucléaire et sont prêtes à entrer en mitose. Dans les cellules traitées, la valeur diploïde moyenne pour les protéines nucléairs

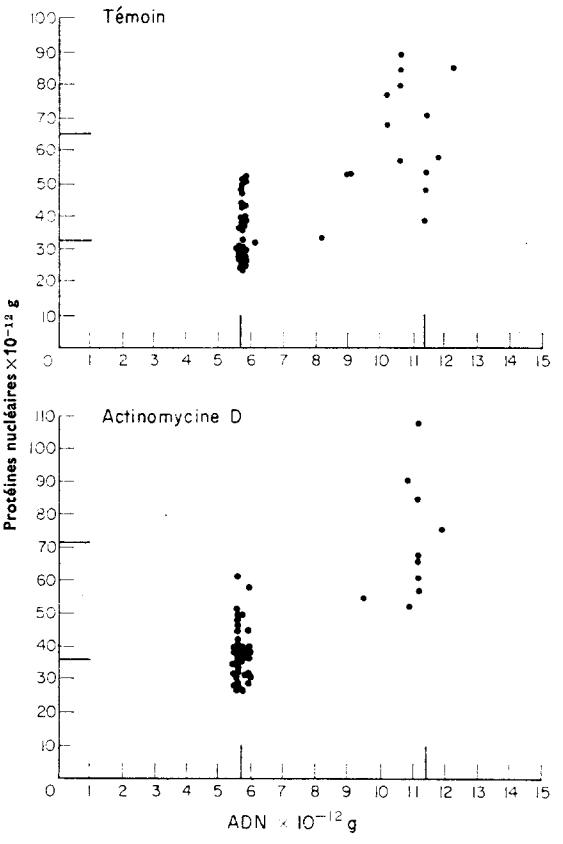


*Fig. 7. Relation entre la teneur en protéines totales et la teneur en ADN dans le noyau de fibroblastes de rat traités par de l'actinomycine D (0·5 µg/ml) et dans les témoins correspondants. Chaque point correspond à un noyau. En abscisses, teneurs en ADN converties en valeurs absolues; en ordonnées, teneurs en protéines nucléaires totales. Les petits traits perpendiculaires aux axes des abscisses ou des ordonnées indiquent les valeurs moyennes pour les noyaux diploïdes ou tétraploïdes. Cinquante et une cellules témoins et 54 cellules traitées ont été étudiées.*

et pour les ADN est quasi la même que chez les témoins. Rares sont les cellules en train de réaliser des synthèses prémitotiques. Environ 18% des fibroblastes ont terminé leurs synthèses et sont en préprophase; cependant, l'activité mitotique est quasi nulle depuis le début de l'expérience. Voici comment nous pensons pouvoir interpréter ces observations. Sous l'effet de l'actinomycine D, les cellules sont bloquées ou restent longtemps en préprophase. Peu d'entre elles entrent en mitose. Notons que parmi les cellules tétraploïdes (4p/4ADN), quelques-unes proviennent de la reconstitution d'une seule cellule mononucléée après une mitose non suivie de cytodiérèse (voir Fig. 2). L'actinomycine D a aussi un certain effet inhibiteur sur les synthèses prémitotiques de matériel nucléaire (protéines totales et ADN); c'est pourquoi le pourcentage de cellules tétraploïdes reste inférieur à celui observé chez les témoins. L'absence (ou le retard) de l'entrée en mitose n'est pas dû ici à une synthèse incomplète de matériel nucléaire

car beaucoup de cellules ont atteint, à ce point de vue, le niveau préprophasique normal. On peut penser que cette inhibition de l'entrée en mitose est liée notamment à la profonde altération des nucléoles qui sont devenus minuscules. La quantité de matériel nucléolaire par noyau est si faible qu'elle n'est pas mesurable avec notre technique interférentielle; chez les témoins, par contre, la teneur en matière sèche nucléolaire est voisine de 7 pg en moyenne au début de l'interphase.

L'actinomycine D est donc capable d'inhiber, dans une certaine mesure, les synthèses prémitotiques de protéines nucléaires totales et d'ADN. Ces résultats obtenus par les méthodes micro-interférentielle et cytophotométrique sont en accord avec ceux fournis par la méthode histo-autoradiographique (voir plus loin). Nos dosages de protéines nucléaires et d'ADN montrent de plus que bon nombre de cellules peuvent atteindre la prépophase, mais peu d'entre elles entrent en mitose.

Dans une seconde expérience, des fibroblastes de rat ont été traités pendant une plus longue durée, mais par une plus faible concentration d'actinomycine D (100 h; 0.025 µg/ml). Des dosages micro-interférentiels et cytophotométriques ont aussi été réalisés dans ces cellules. Les résultats obtenus sont en accord avec ceux de la première expérience. En effet, l'activité mitotique est quasi nulle (0,5 °/°°), mais il existe des cellules en prépophase (environ 16%). Ces cellules sont donc bloquées en post-synthèse. Une certaine inhibition s'exerce sur les synthèses de protéines et d'ADN car ce pourcentage de cellules tétraploïdes n'est pas élevé. L'inhibition est cependant moins forte que pour une concentration de 0,5 µg/ml d'actinomycine D; en effet, il existe ici un plus grand nombre de cellules à teneur intermédiaire, c'est-à-dire en train de réaliser la duplication de leur teneur en ADN et en protéines nucléaires. La taille des nucléoles est réduite, mais dans une moindre mesure qu'après un traitement par de l'actinomycine à 0,5 µg/ml. Ici, les nucléoles ont une surface de projection de 1 à 2 $\mu^2$ (contre 3 à 12 $\mu^2$ et même 20 $\mu^2$ chez les témoins). Leur poids sec a été mesuré par microscopie interférentielle. Il est situé entre 1 et 4 pg (valeur totale par noyau). Chez les témoins, les valeurs se situent entre 5 et 16 pg; ces variations du poids sec nucléolaire dans les cellules non traitées sont en relation avec l'existence de synthèses de protéines nucléolaires au cours de l'interphase. C'est le phénomène que nous avons appelé la "préparation nucléolaire à la mitose" [94]. La diminution du volume nucléolaire observée dans les cellules traitées s'explique donc par une importante perte de matière sèche nucléolaire.

3. *Etude histo-autoradiographique.*—L'incorporation de thymidine, d'uridine ou de leucine tritiées dans des fibroblastes de rat, traités par de l'actinomycine D, a été étudiée par la méthode histo-autoradiographique.

(*a*) *Incorporation de thymidine tritiée.*—Lorsque la thymidine tritiée est ajoutée en même temps que l'actinomycine D (0,5 µg/ml), on constate, après 24 h, que 40% des cellules ont incorporé du précurseur contre 95% chez les témoins. Si la thymidine tritiée est ajoutée au milieu nutritif de cellules préalablement traitées pendant 24 h par de l'actinomycine D (0,5 µg/ml), 19% seulement des cellules sont marquées, mais très faiblement, après 3 h 30 de contact; chez les témoins correspondants, 34% des cellules sont très marquées (Fig. 8). Ces résultats montrent donc que l'actinomycine D est capable d'inhiber les synthèses d'ADN, mais ils montrent également qu'une certaine incorporation de thymidine peut encore avoir lieu dans les cellules même après 24 h de traitement. Ces résultats sont en bon accord avec ceux que nous avons obtenus par la méthode cytophotométrique.

Dans une autre condition expérimentale, de la thymidine tritiée a été ajoutée au milieu de culture après 72 h de traitement par de l'actinomycine D à une faible concentration (0,025 µg/ml). Après 3 h 30 de contact avec le milieu nutritif contenant à la fois du précurseur tritié et de l'actinomycine D, 1,6% seulement des cellules sont marquées, et très faiblement, contre 8% chez les témoins où le marquage est beaucoup plus intense*. L'effet inhibiteur qu'exerce l'actinomycine D sur les synthèses d'ADN apparaît donc ici aussi très nettement.

(*b*) *Incorporation d'uridine tritiée.*—Lorsque de l'uridine tritiée est ajoutée au milieu de culture avec l'actinomycine D (0,5 µg/ml) et reste au contact des cellules pendant 24 h, le marquage de l'ensemble de la cellule est faible, alors qu'il est intense dans les cellules témoins. Chez ces dernières, et conformément aux

*On remarquera que le pourcentage de cellules ayant incorporé de la thymidine tritiée chez les témoins est relativement faible (8%) par rapport aux pourcentages observés dans les cultures témoins de l'expérience précédente (34%), alors que le temps de contact avec le précurseur avait été de 3 h 30 dans les deux cas. Dans le ler cas, les cultures étaient en effet en fin de croissance car le dernier entretien avait été effectué 72 h plus tôt. Dans le second cas, par contre, la croissance des cultures était plus active car elles avaient été entretenues 24 h auparavant.

notions classiques [95], les nucléoles et le cytoplasme sont intensément marqués alors que la chromatine l'est nettement moins. Au niveau des cellules traitées, par contre, les grains d'argent sont uniformément distribués sans marquage prépondérant d'aucun élément cellulaire. Lorsque l'uridine tritiée est ajoutée à des cultures préalablement traitées pendant 24 h par de l'actinomycine D (0,5 µg/ml), nous observons, après 3 h 30 de contact avec le précurseur et l'actinomycine, que les cellules ne sont pas marquées. Chez les témoins, par contre, toutes les cellules ont incorporé de l'uridine tritiée, et de façon particulièrement intense au niveau des nucléoles (Fig. 9).

Nous avons aussi ajouté de l'uridine tritiée au milieu de culture après 72 h de traitement par de l'actinomycine D à faible concentration (0,025 µg/ml); les cellules sont restées au contact de ce précurseur pendant 3 h 30 en présence d'actinomycine D. Dans ces conditions, on n'observe que de très rares grains d'argent au niveau du cytoplasme et du noyau. Par contre, toutes les cellules témoins sont nettement marquées.

Ces résultats montrent clairement que l'actinomycine D exerce un puissant effet inhibiteur sur les synthèses d'ARN dans notre matériel.

(*c*) *Incorporation de leucine tritiée.*—Si la leucine tritiée est ajoutée au milieu de culture en même temps que l'actinomycine D (0,5 µg/ml), et si la durée de contact est de 24 h, on ne note pas de modification appréciable dans l'intensité du marquage des cellules par rapport à celui observé chez les témoins. Par contre, si la leucine tritiée est ajoutée au milieu nutritif de cultures traitées depuis 24 h par l'actinomycine D (0,5 µg/ml), après 3 h 30 de contact avec le précurseur et l'actinomycine D, les cellules sont faiblement marquées alors que les cellules témoins le sont intensément (Fig. 10). Les synthèses protéiques sont donc elles aussi inhibées par l'actinomycine D; cette inhibition semble toutefois se produire plus tardivement que celle des synthèses d'acides nucléiques.

De la leucine tritiée a aussi été ajoutée au milieu de culture après 72 h de traitement par de l'actinomycine D (0,025 µg/ml). Après 3 h 30 de contact avec ce précurseur et l'actinomycine D, on n'observe pas de différence dans l'intensité du marquage par rapport aux témoins. Les synthèses de protéines ne sont donc probablement pas inhibées, ou ne le sont que très peu dans ces dernières conditions alors que les synthèses d'acides nucléiques le sont nettement.

*C. Localisation de l'actinomycine D dans la cellule*

Il a été possible de déterminer l'endroit où se fixe l'actinomycine D dans la cellule grâce à l'utilisation d'actinomycine D tritiée. Des fibroblastes de rat vivants ont été mis au contact du produit pendant 2 h. Dans les histo-autoradiographies, on constate que le noyau est très marqué dans 100% des cellules, mais que le cytoplasme ne l'est pas.

Le même résultat est obtenu si on procède de la même manière avec des fibroblastes fixés (Fig. 11). Le marquage du noyau est intense dans les deux cas et il disparaît après une digestion par la désoxyribonucléase neutre (Fig. 11). L'actinomycine D se fixe donc bien sur l'ADN, comme d'autres auteurs l'ont montré aussi pour d'autres matériels.

## DISCUSSION

Beaucoup de travaux ont été consacrés à l'étude des effets de l'actinomycine D sur des matériels très variés. Le présent travail a été entrepris dans le cadre de recherches concernant les effets d'agents antimitotiques sur des cellules cultivées *in vitro* (voir notamment [82, 96]). Nous avons étudié des cellules traitées par de l'actinomycine D à l'aide d'un large éventail de techniques cytologiques et cytochimiques. Ces recherches ont été effectuées dans des fibroblastes de poulet ou de rat cultivés *in vitro*. Ces différentes méthodes nous ont fourni des résultats concordants. A notre connaissance, très peu d'auteurs avaient utilisé jusqu'ici des techniques telles que les examens en contraste de phase sur le vivant et les dosages par cytophotométrie et microscopie interférentielle pour analyser les effets de l'actinomycine D. D'une manière générale, nos conclusions sont en accord avec celles obtenues par d'autres auteurs, mais ici plusieurs faits nouveaux ont été observés.

Les altérations nucléolaires provoquées par l'actinomycine D ont fait l'objet de nombreux travaux (voir l'Introduction). Grâce à l'observation en contraste de phase *sur le vivant*, nous avons pu observer en détail la fragmentation et la condensation des nucléoles. Lors de la fonte du nucléole, la chromatine périnucléolaire reste bien visible dans les cellules fixées et colorées; ceci est en accord avec les observations de Caspersson *et al.* [48]. Après coloration au bleu de toluidine, il est possible de distinguer 2 zones dans les nucléoles des cellules traitées: une zone bien colorée et une zone incolore. Des images analogues ont été observées par Seite [14] dans des neurones ganglionnaires de rat traités par de l'actinomycine D. Ces altérations nucléolaires ont été décelées grâce

au microscope optique. Ces observations sont à rapprocher de celles réalisées au microscope électronique par différents auteurs qui ont étudié les modifications des nucléoles provoquées par l'actinomycine D dans des matériels très variés. Les profonds remaniements subis par ces nucléoles ont été décrits dans l'introduction du présent travail. Nous avons constaté, dans notre matériel, que l'incorportation d'uridine dans les ARN du nucléole est fortement inhibée par l'actinomycine D, en accord avec les observations d'autres auteurs (voir notamment [50, 56, 57] ). Pour notre part, nous avons montré en plus que le poids sec nucléolaire total par noyau est fortement diminué sous l'effet de l'actinomycine D. La surface de projection des ces nucléoles est également très réduite. La diminution de la taille des nucléoles s'explique donc par une importante perte de matière sèche nucléolaire. Lorsque le traitement est de longue durée, et lorsque la concentration en actinomycine D dans le milieu de culture est élevée, les nucléoles deviennent si petits qu'ils no sont plus mesurables par notre méthode interférentielle.

En général, la taille du noyau n'est pas modifiée par l'actinomycine D. Dans notre matériel, et en particulier chez les fibroblastes de poulet, nous avons constaté que, sous l'effet du traitement, le noyau peut prendre un aspect "granulo-filamenteux", pseudo-prophasique, comparable à celui décrit par Chèvremont *et al.* [97] pour des fibroblastes traités *in vitro* par de la ribonucléase. Après traitement par de l'actinomycine D, des modifications nucléaires assez semblables ont été observées par Clark *et al.* [13] dans des cellules HeLa, par Mittermayer *et al.* [12] dans des cellules L et par Fautrez–Firlefyn et Fautrez [26] chez *Artemia salina.* Nous avons montré que, dans notre matériel, cet aspect pseudo-prophasique est lié à une dégénérescence du noyau qui peut se déclencher à n'importe quel moment du cycle cellulaire et n'est donc pas liée à un blocage en préprophase. Pour Melvin [36] cependant, ce serait la durée de la prophase qui serait allongée dans le foie de souris en régénération, traité par de l'actinomycine D. Il est très intéressant de constater que la ribonucléase [97] provoque une dégénérescence granulo-filamenteuse du noyau, comparable à celle que nous avons observée dans notre matériel après un traitement par de l'actinomycine D. De plus, après action de la ribonucléase sur des fibroblastes cultivés *in vitro*, les nucléoles sont également touchés. Ils se fragmentent en petites masses arrondies [97]. Robineaux *et al.* [98] ont observé une modification ultrastructurale des nucléoles après action de la ribonucléase; ceux-ci présentent une "ségrégation" de leurs constituants, assez semblable à celle provoquée par l'actinomycine D. Or, sous l'effet de la ribonucléase [95] comme sous 'effet de l'actinomycine D, les synthèses d'ARN sont fortement diminuées. Ce fait pourrait être de nature à expliquer pourquoi les lésions cytologiques provoquées par ces deux antimitotiques sont assez semblables. Soulignons cependant que l'actinomycine D se fixe sur la molécule d'ADN ( [45] et d'autres), alors que la ribonucléase agit directement sur l'ARN.

Dans notre matériel, l'activité mitotique est fortement inhibée par l'actinomycine D dès les premières heures du traitement. Ce fait avait déjà été démontré dans divers matériels par plusieurs auteurs [27–29]. Toutefois, à notre connaissance, aucun travail n'avait été consacré à l'étude du déroulement de la mitose dans des cellules traitées par l'actinomycine D et observées sur le vivant. Dans nos cultures traitées, de rares mitoses se produisent; la durée en est souvent allongée. La métaphase peut durer jusqu'à 21 h, mais la cellule finit par se diviser de façon normale ou présente des troubles de la cytodiérèse. Il se reforme alors une cellule à gros noyau unique ou à deux noyaux de taille habituelle. Nous n'avons pas observé de cassures de chromosomes ni de chromosomes aberrants, contrairement à Arrighi et Hsu [37], Ostertag et Kersten [38], Fraccaro *et al.* [10] qui ont étudié les effets de l'actinomycine D sur des cellules animales cultivées *in vitro.* Soulignons que les troubles de la division cellulaire, provoqués dans notre matériel par l'actinomycine D, ne sont pas spécifiques et peuvent être observés dans des cellules traitées par des agents antimitotiques variés (voir notamment, [96]).

Dans nos conditions expérimentales, l'actinomycine D inhibe fortement les synthèses d'ARN. L'incorporation d'uridine tritiée dans les ARN est très faible ou nulle dans les fibroblastes traités; cette inhibition se marque aussi bien au niveau de la chromatine et des nucléoles qu'au niveau du cytoplasme. Ces faits sont en parfait accord avec les observations de nombreux auteurs cités dans l'introduction et qui avaient étudié des espèces cellulaires très variées soumises à l'action de l'actinomycine D *in vivo* ou *in vitro.* Rappelons qu'il est bien admis actuellement que l'actinomycine D se fixe sur l'ADN et entraîne de ce fait une inhibition de la synthèse des ARN [29, 45, 62].

Jusqu'ici, rares sont les auteurs qui ont étudié les effets de l'actinomycine D sur la

teneur en ADN du noyau par la méthode cytophotométrique après réaction de Feulgen. Rappelons que l'intérêt principal de cette méthode réside dans le fait qu'elle permet de déterminer la teneur en ADN du noyau dans des cellules considérées individuellement. Nous avons constaté que, dans notres matériel, la *teneur diploïde* en ADN n'est pas modifiée par l'actinomycine D, du moins dans les limites de précision de nos mesures. En ce qui concerne les synthèses d'ADN, nos observations ont été réalisées non seulement par cytophotométrie, mais également par la méthode histo-autoradiographique basée sur l'incorporation de thymidine tritiée. Grâce à ces deux méthodes, nous avons montré que les *synthèses* d'ADN sont inhibées par l'actinomycine. Toutefois, dans nos conditions expérimentales, cette inhibition n'est pas totale mais elle est beaucoup plus intense lorsque le traitement est appliqué à des cultures en fin de croissance. Parmi d'autres, Reich [66], Prudhomme *et al.* [67], Soriano [68], Kim *et al.* [69] ont également observé que l'actinomycine D inhibe les synthèses d'ADN dans les cellules animales *in vivo* ou *in vitro*. Pour Caspersson *et al.* [48], les synthèses d'ADN seraient inhibées plus tardivement que les synthèses d'ARN et de protéines dans des cellules de souris traitées *in vitro* par de l'actinomycine D. Dans nos conditions expérimentales, certains fibroblastes parviennent à atteindre la préprophase, c'est-à-dire doublent leur teneur en ADN (4 ADN). Toutefois, la plupart de ces cellules sont bloquées à ce stade du cycle cellulaire.

Grâce à la microscopie interférentielle, nous avons déterminé la teneur en protéines nucléaires totales dans des fibroblastes traités par l'actinomycine D et considérés individuellement. A notre connaissance, de telles mesures n'avaient pas encore été réalisées. Nous avons montré ainsi que, dans les cellules bloquées en post-synthèse, la teneur en protéines totales du noyau est tétraploïde comme la teneur en ADN. Dans ces cellules, les teneurs en ADN et en protéines nucléaires sont donc au niveau préprophasique normal, c'est-à-dire sont, à ce point de vue, capables d'entrer en mitose [82]. Mais dans ces cellules en préprophase, les nucléoles sont très altérés; nous avons montré grâce à la méthode interférentielle, que la quantité de matériel nucléolaire par noyau est fortement diminuée et parfois quasi nulle. Nous pensons que ce fait est de nature à expliquer pourquoi ces cellules, quoique tétraploïdes, sont incapables d'entrer en prophase. En effet, l'un de nous a montré antérieurement [94] que, lors de la préparation à la mitose, la quantité de matériel nucléolaire double, comme c'est aussi le cas pour les protéines nucléaires et les ADN. Il semble donc bien que la lésion profonde des nucléoles puisse être au moins l'un des facteurs responsables de l'absence d'entrée en mitose. Nos observations sont en accord avec celles de Goldstein *et al.* [61]. En effet, ces auteurs ont montré par cytophotométric après réaction de Feulgen que les cellules HeLa traitées par de l'actinomycine D (1 µg/ml pendant 24 h) sont capables de synthétiser des ADN mais elles ne peuvent se diviser. Dans l'oeuf *d'Artemia salina* en segmentation [26], les cellules sont capables de réaliser leurs synthèses d'ADN préparatoires à la mitose malgré un traitement par de l'actinomycine D mais elles ne peuvent entrer en mitose; ces faits sont également assez analogues à ceux que nous avons observés dans notre matériel.

Grâce à la méthode histo-autoradiographique basée sur l'incorporation de leucine tritiée, nous avons constaté que l'actinomycine D est capable d'inhiber dans une certaine mesure les synthèses de protéines *cellulaires*, comme d'autres auteurs l'avient montré aussi pour différents matériels. Toutefois, dans nos conditions expérimentales, cette inhibition semble se produire plus tardivement que celle des synthèses d'acides nucléiques. Quant aux résultats de nos dosages micro-interférentiels, ils ont montré que les synthèses de protéines *nucléaires* totales subissent une certaine inhibition. Ces deux méthodes donnent donc des résultats tout à fait concordants. Comme nous l'avons rappelé plus haut, certaines cellules sont cependant capables de réaliser la duplication prémitotique de leur teneur en protéines nucléaires. Lorsque l'actinomycine D est utilisée à une faible concentration, les synthèses d'acides nucléiques peuvent être inhibées alors que l'incorporation de leucine tritiée ne l'est pas.

En traitant des cellules vivantes par de l'actinomycine D *tritiée*, nous avons montré par histo-autoradiographie que cet antimitotique se fixe sur les ADN du noyau, en accord avec les observations de Harbers *et al.* [73], Fraccaro *et al.* [10], Ro *et al.* [56], Rothstein *et al.* [75] et Simard [78] notamment. Nous avons également traité par de l'actinomycine D tritiée des cellules préalablement fixées. Nous avons constaté aussi une localisation élective au niveau du noyau. Cette dernière méthode a été mise au point par Brachet et Ficq [79, 80] et Brachet et Preumont [81]; elle permet de détecter spécifiquement les ADN dans des cellules fixées.

Selon Goldstein *et al.* [61], les cellules HeLa fortement altérées par un traitement par de l'actinomycine D (0,1 µg/ml pendant 48 h) sont capables de se multiplier à nouveau après lavage et retour en milieu nutritif dépourvu d'actinomycine D. De même, les effets de l'actinomycine D sur l'activité mitotique et sur l'incorporation de précurseurs d'ARN dans des cellules végétales sont *réversibles* dans certaines conditions expérimentales [53, 58]. Clark *et al.* [13] ont montré que si on lave des cultures de cellules HeLa qui avaient été traitées par une faible concentration d'actinomycine D (0,01 µg/ml), on constate que l'activité mitotique, fortement inhibée, reprend au bout de 2 jours lorsque les cultures sont replacées dans un milieu nutritif dépourvu d'actinomycine. De leur côté, Oda et Chiga [22] et Goldblatt *et al.* [23] ont constaté que les nucléoles du foie de rat, qui présentaient une ségrégation de leurs constituants suite à un traitement par de l'actinomycine D, peuvent retrouver, après un certain temps, une configuration ultrastructurale normale. Pour notre part, nous avons observé dans notre matériel que les lésions produites par l'actinomycine D, dans certaines conditions expérimentales, peuvent être réversibles. Ces expériences sont actuellement en cours. Voici en résumé quelles sont nos observations préliminaires [87]; ces dernières seront développées en détail ultérieurement [99]. Des fibroblastes qui avaient été traités par de l'actinomycine D (0,025 µg/ml), pendant 48 h, ont été lavés, puis remis en présence de milieu nutritif dépourvu d'actinomycine D; 48 h après ce lavage, l'activité mitotique, qui était fortement inhibée, reprend de façon nette et les nucléoles retrouvent une taille quasi identique à celle des témoins. L'incorporation de thymidine et celle d'uridine tritiées, qui avaient été profondément inhibées pendant le traitement redeviennent également normales. Après ce traitement par l'actinomycine D, et avant le lavage, nous avons constaté que les nucléoles présentent, au microscope électronique, une nette "ségrégation" de leurs constituants, en accord avec différents auteurs cités plus haut. Suite au lavage, les nucléoles de nos fibroblastes peuvent retrouver une structure submicroscopique normale quoique, dans certains cas, des signes de ségrégation persistent mais tendent à disparaître. Ces observations nous paraissent importantes car elles suggèrent en fait que l'une des conditions indispensables pour que la cellule puisse entrer en mitose est que les nucléoles soient fonctionnels et présentent une structure normale. En effet, nous avons constaté que les cellules traitées, puis lavées, dont les nucléoles ont retrouvé une taille et une configuration ultrastructurale normales se remettent à se multiplier. De tels faits sont de nature à supporter la notion défendue pa l'un de nous [82] selon laquelle une des étapes capitales de la préparation à la mitose consiste en une préparation nucléolaire au cours de laquelle la quantité de matière sèche nucléolaire est doublée, comme c'est aussi le cas pour les protéines et les ADN du noyau.

On peut se demander si la fixation de l'actinomycine D sur les ADN du noyau n'est pas responsable de modifications des propriétés de cet acide nucléique. Outre l'inhibition bien connue des synthèses d'ARN, ces modifications pourraient être telles que, d'une part, dans beaucoup de cellules, les ADN ne peuvent réaliser leur duplication et que, d'autre part, dans les fibroblastes arrivés en préprophase, la formation des chromosomes est empêchée. La dégénérescence granulo-filamenteuse du noyau, observée aussi dans notre matériel, pourrait être due également à des altérations de la chromatine. La liaison ADN-actinomycine paraît cependant relativement labile puisque, dans certaines conditions, après lavage, les synthèses d'ADN et d'ARN reprennent et les mitoses se déclenchent.

## RESUME

*Les effets de l'actinomycine D sur des fibroblastes de poulet ou de rat cultivés* in vitro *ont été étudiés à l'aide d'un large éventail de techniques cytologiques et cytochimiques.*

*Sous l'effet de l'actinomycine D, la taille des nucléoles diminue considérablement dès les premières heures du traitement. Ce fait a été démontré dans d'autres matériels par de nombreux auteurs. Des examens en contraste de phase sur le vivant nous ont permis de constater que l'actinomycine D peut provoquer un allongement de la durée de la métaphase et l'inhibition de la cytodiérèse. Comme d'autres auteurs l'avaient montré, l'actinomycine D est aussi un puissant inhibiteur de l'activité mitotique.*

*La teneur en ADN de noyaux interphasiques ou de figures mitotiques a été déterminée par cytophotométrie après réaction de Feulgen. La teneur diploïde en ADN n'est pas modifiée par l'actinomycine D. Celle-ci provoque une inhibition des synthèses prémitotiques*

*d'ADN, comme d'autres auteurs l'avaient montré aussi pour d'autres types cellulaires. En outre, selon nos observations, certaines cellules peuvent cependant devenir tétraploïdes quant aux ADN mais sont bloquées à ce stade (post-synthèse ou G2). Sous l'effet de l'actinomycine D, les noyaux peuvent devenir granulo-filamenteux: il s'agit d'une dégénérescence nucléaire qui peut atteindre les cellules à n'importe quel moment du cycle cellulaire.*

*La teneur en protéines totales du noyau et le poids sec total du matériel nucléolaire par noyau ont été déterminés par microscopie interférentielle dans des fibroblastes de rat cultivés* in vitro *en présence d'actinomycine D. Ensuite, dans les mêmes cellules préalablement repérées, la teneur en ADN a été mesurée par cytophotométrie après réaction de Feulgen. Ces mesures nous ont permis de montrer que les synthèses prémitotiques de protéines nucléaires et d'ADN sont inhibées, mais pas complètement; certaines cellules sont capables de doubler leur teneur en protéines nucléaires totales et en ADN, c'est-à-dire d'atteindre la préprophase. La plupart de ces cellules ne peuvent cependant pas entrer en mitose, vraisemblablement parce que les nucléoles sont très altérés. En particulier, le poids sec nucléolaire total par noyau est fortement diminué ou quasi nul.*

*Par la méthode histo-autoradiographique, nous avons montré que les synthèses d'ADN, d'ARN et de protéines cellulaires peuvent être inhibées par l'actinomycine D. Ces faits ont été observés par plusieurs auteurs dans d'autres matériels. Nous avons constaté aussi qu'à faible concentration, l'actinomycine D est capable d'inhiber les synthèses d'acides nucléiques alors que des synthèses protéiques se poursuivent.*

*En accord avec quelques auteurs, nous avons montré par histo-autoradiographie que l'actinomycine D tritiée se fixe sur l'ADN du noyau.*

*Selon des résultats d'expériences en cours, les effets de l'actinomycine D peuvent être réversibles dans certaines conditions expérimentales.*

## SUMMARY

*The effects of actinomycin D on chick or rat fibroblasts cultivated* in vitro *have been studied by several cytological and cytochemical methods.*

*A few hours after the beginning of treatment, the size of the nucleoli diminishes considerably as already shown by others for other materials. Living treated cells have been observed by phase contrast microscopy. The duration of metaphase is increased and the separation of daughter-cells at telophase is often inhibited. The mitotic activity is strongly depressed.*

*The DNA content has been measured by cytophotometry after Feulgen reaction in individual cells during interphase or mitosis. The diploid DNA content is not modified by actinomycin D. The latter inhibits premitotic DNA synthesis as also shown by others. Furthermore we have shown that some cells become tetraploid as far as the DNA content is concerned but are blocked as this stage (G2 or post-synthesis). Due to the treatment, some nuclei become granulo-filamentous; this nuclear degeneration can start at any moment of the cellular cycle.*

*The nuclear total protein content and the nucleolar total dry mass per nucleus have been measured by micro-interferometry in rat fibroblasts. The DNA content has then been measured in the same cells by cytophotometry after Feulgen reaction. Premitotic nuclear protein and DNA synthesis is inhibited but not completely; some cells reach the preprophase (tetraploid nuclear total protein and DNA content). However, the majority of these cells cannot enter into morphological mitosis probably because the nucleoli are altered. In this respect, we have shown that the nucleolar total dry mass per nucleus is strongly reduced.*

*By histo-autoradiography, we have observed that actinomycin D inhibits DNA, RNA and protein synthesis, as also shown by others. According to our results, in cells treated by low concentrations of actinomycin D, nucleic acids synthesis can be inhibited but protein synthesis remains normal. We have also shown by histoautoradiography that tritiated actinomycin D is linked to DNA in the nucleus.*

*According to the results of experiments now in progress, cells treated by actinomycin D can recover under some conditions.*

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# Pulmonary Tumour Induction *in vitro* Genetic Influence

ANTONIA FLAKS

*Department of Experimental Pathology and Cancer Research, School of Medicine, Leeds, England*

THAT tumours could be induced in pulmonary tissue which had been incubated *in vitro* with a carcinogen and subsequently implanted into isogeneic mice has been demonstrated by Flaks and Laws [1, 2]. In those experiments, the tissue had been obtained from Balb/c mice, a strain which is genetically susceptible to pulmonary-tumour formation. The present investigation was designed to assess the influence of genetic susceptibility to *in vivo* tumour formation on the interaction of carcinogen and pulmonary tissue *in vitro*, without the involvement of general homeostatic mechanisms. For this purpose, pulmonary tissue was taken from mice of the C57Bl strain, which is genetically resistant to the formation of pulmonary neoplasms.

## MATERIAL AND METHODS

C57Bl mice, bred in this laboratory by strict brother-sister mating with frequent reselection of lines, were fed on Oxoid 41B diet and given water *ad libitum*.

Pulmonary tissue, removed from 1 month-old females, was cultured *in vitro* by the technique used in previous experiments [1, 2]. Each explant was approximately $2 \times 1 \times 1$ mm in size and fifteen explants were maintained in each Trowell's chamber in Trowell's medium [3]. The carcinogenic medium was prepared by the addition of 4 μg of 20-methylcholanthrene to 1 ml of medium. The lung explants were gassed with 95% $O_2$ and 5% $CO_2$ for 10 min daily at a rate of 75 ml/min. They were maintained in either normal or carcinogenic medium for one day, then the latter were transferred into normal medium for a further day to minimize the possibility of transference of free carcinogen to the host at the time of implantation.

Twenty-four mice received subcutaneous implants of pulmonary tissue incubated in carcinogen containing medium while a similar number of animals were implanted with tissues which had been kept in normal medium. At 3, 6, 9 and 12 months after implantation, a number of mice from each group were autopsied and the implants removed, fixed in 10% formol-saline, embedded in paraffin-wax, serially sectioned at 5 μ and stained with haematoxylin and eosin for histopathological examination.

## RESULTS

Microscopical examination of a number of explants immediately after *in vitro* culture in either carcinogenic or normal medium revealed only mild necrosis and slight lymphocytic hyperplasia, the structure of the tissue being otherwise normal, although the lungs were collapsed.

The implants which had been previously cultured in normal medium, when examined at any of the stated times after implantation, exhibited a normal, well preserved pulmonary architecture, apart from moderate patches of lymphocytic hyperplasia and some cystic dilatation of the bronchioli. However, implants which had been exposed to 20-methylcholan-

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threne *in vitro* showed the presence of an extensive peribronchiolar and perivascular lymphocytic reaction. In some cases this replaced the lung tissue and gave the implant a lymph node-like appearance. The degree of lymphocytic hyperplasia in the control implants from the normal medium served as a baseline for the evaluation of the lymphocytic hyperplasia in the carcinogen treated implants. The difference between the two groups of implants was readily apparent and the results were confirmed by an independent pathologist. No tumours developed during the entire course of the experiment. Some degree of fibrosis affected all implants. No abnormality was detected in any organ of the host animals and the tissues contiguous to the implant were unaltered.

## DISCUSSION

Previous experimental work involving murine pulmonary carcinogenesis *in vivo* [4,5] demonstrated that the degree of genetic susceptibility or resistance to carcinogenesis is controlled by factors which are intrinsic to the target organ. The present investigation has shown that this property persists and functions *in vitro* when pulmonary tissue is in contact with a carcinogen, in the absence of organismal-tissue-homeostatic mechanisms, and has confirmed the hypothesis that genetic factors exercise a dominant role in the *in vitro* induction of murine pulmonary cancer. It is clear that the results of *in vitro* experiments in carcinogenesis will be influenced, in the first instance, by the degree of susceptibility of the donor animals, and that this should be taken into consideration in choosing the strain of mice to be used in this type of investigation.

Laws and Flaks [1] suggested that, in pulmonary implants, lymphoid elements grew more rapidly than did other cellular components and that such a response may have resulted from the abnormal anatomical situation of the implanted tissue. In addition, implants derived initially from tumour-susceptible donors, whether incubated in normal or carcinogen-added media, displayed a similar degree of lymphocytic hyperplasia. In the present experiment which utilized tumour-resistant donors, however, implants which had been incubated in a carcinogen-containing medium developed a considerably greater lymphocytic reaction than did those maintained in the normal medium. The presence of such cellular response in C57Bl pulmonary tissue implants may be indicative of the activity of 20-methylcholanthrene as a cancer-inducing agent; it is possible that an antigenic change may have taken place, despite the fact that adenomat did not develop, and the proliferative reaction may have been the result of the defensive action of tissue-regulating mechanisms. The fact that neither the organs nor the tissues adjacent to the pulmonary implants in the host showed any abnormality suggests that the latter did not influence the behaviour of the implants.

**Acknowledgements** — I wish to thank the pathologist, Dr. J. M. Hamilton, for his help in the evaluation of the results.

## SUMMARY

*Pulmonary tissues, taken from C57Bl mice, were incubated* in vitro *for one day in 20-methylcholanthrene medium and subsequently implanted into animals of the same strain. Histopathological examination of the implants at periods varying between 3 and 12 months showed the presence of intense lymphocytic hyperplasia but the total absence of adenomata. As C57Bl mice are known to be genetically resistant to the* in vivo *formation of pulmonary neoplasms and as no neoplasms were induced by the* in vitro *method, it was considered that tissue-specific genetic factors exercised a dominant role in the activity of the carcinogen.*

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# Subcellular Particles in Tumors—III

## Peroxisomal Enzymes in Hepatoma HC and Morris Hepatomas 7794A, 7794B, 5123A and 7316A

R. WATTIAUX*, S. WATTIAUX-DE CONINCK*, J. M. VAN DIJCK*, and H. P. MORRIS†

**Laboratory of Physiological Chemistry, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium, and*

*†Department of Biochemistry, Cancer Research Unit, College of Medicine, Howard University, Washington, D.C., U.S.A.*

### INTRODUCTION

PEROXISOMES are subcellular particles first identified in rat-liver tissue by de Duve and his associates [1, 2]. Several biochemical investigations have subsequently shown their presence in rat kidney [3–5], protozoa [4, 6] and plant tissue [7]. In rat liver, they are enzymatically characterized by catalase and hydrogen peroxide forming oxidases acting on D-amino acids, Lα hydroxy-acids and urate; morphologically they correspond to the "microbodies" [8]. At the present time, little information is available concerning these organelles in neoplastic cells. Ultrastructural observations of Dalton [9] indicate that the microbodies are present in some slowly growing Morris hepatomas but are not visible in a rapidly growing tumor. On the other hand, numerous studies have been performed on tumor catalase but without considering the intracellular localization of the enzyme. As we have shown [10, 11], the catalase in transplantable hepatoma HW, being much less active than the rat-liver catalase, exhibited a structure-linked latency and sedimented with the microsomal fraction. Moreover, neither D-amino acid oxidase nor urate oxidase were measurable in that tumor. In a systematic study by centrifugation methods of subcellular structures in Morris hepatomas, our attention has been primarily focused on "peroxisomal" enzymes of these tumors. The results presented here have been obtained on five transplantable hepatomas: four medium to slowly growing tumors (7794A, 7794B, 5123A, 7316A) and one, HC, a rapidly growing one. The results record the activity of catalase, D-amino acid oxidase and urate oxidase found in the homogenates of these tumors and show how these enzymes are distributed after differential and isopycnic centrifugation. They are discussed in respect to the presence of peroxisomes in the tumors and their properties.

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### MATERIAL AND METHODS

*Tumors*

Morris hepatomas 7316A, 7794A, 7794B and 5123A are moderately slowly and slowly growing tumors as measured by the time between transplantations [12, 13]. They all show well differentiated trabecular patterns [14]. HC hepatoma is a more rapidly growing trabecular carcinoma [12, 15]. The tumor-bearing animals are shipped by air express from Washington D.C. and maintained in our laboratory in Namur, Belgium, until use.

*Tissue fractionation*

The rats were sacrificed by decapitation, the tumor was rapidly dissected, cut in small

*Table* 1. *Enzymatic activities of hepatomas. Values are given in unit/*100 *mg proteins. The results are presented as means* ±*S.D. In parentheses, the values estimated from the data obtained by Baudhuin* et al. [1] *on normal rat liver by the same techniques*

| Hepatoma | HC | 7316A | 5123A | 7794B | 7794A | Liver |
|---|---|---|---|---|---|---|
| No. of experiments | 3 | 3 | 4 | 4 | 4 | 4 |
| Catalase | 21·6±1·4 | 18·9±0·4 | 2·80±0·5 | 9·7±2·7 | 25·0±4·4 | 20·7±0·3 (22·4) |
| D-amino acid oxidase | 0 | 0·53±0·07 | 0·20±0·02 | 0·29±0·11 | 0·02±0·01 | 0·208±0·016 (0·320) |
| Urate oxidase | 0 | 0·93±0·13 | 1·30±0·25 | 1·34±0·39 | 0 | 1·40 ±0·11 (1·66) |

pieces on a refrigerated Petri dish and put in ice-cold 0·25M sucrose. Homogenization was performed with a tissue grinder fitted with a Teflon pestle rotating at 3000 rev/min (Arthur Thomas, Philadelphia, U.S.A.). Fractionation of the homogenate by differential centrifugation was achieved using the technique of de Duve *et al.* [16]. A nuclear fraction N, a "heavy" mitochondrial fraction M, a "light" mitochondrial fraction L, a microsomal fraction P and a soluble fraction S were isolated using the fractionation scheme described by these authors.

In density gradient centrifugation experiments, a washed total mitochondrial fraction, corresponding to the sum of fractions M and L was used. Experiments were performed according to Beaufay *et al.* [2]. The Spinco centrifuge was not equipped with the special device used by these authors to ensure slow acceleration and deceleration and to stabilize the rotor at low speed. However, to prevent wobbling of the rotor (SW39), the rotor was damped manually during the first moments of acceleration and at the end of the run.

*Enzyme assays*

Catalase, D-amino acid oxidase and urate oxidase were determined according to Baudhuin *et al.* [1] and proteins were assayed by the method of Lowry *et al.* [17] with bovine serum albumin as standard. According to Baudhuin *et al.* [1] one catalase unit corresponds to the amount of enzyme causing the destruction of 90% of the substrate in 1 min in a volume of 50 ml under the assay conditions. One unit of D-amino acid oxidase activity is defined as the amount of enzyme forming 1 μmole of pyruvate/min under the conditions described. For urate oxidase, one unit of enzymic activity is the amount of enzyme able to oxidize one μmole of urate per min.

## RESULTS

*Enzymatic activities*

Table 1 indicates the mean activities of catalase, D-amino acid oxidase and urate oxidase in the five hepatomas. It also allows a comparison between these activities and the ones found in the normal rat liver. D-amino acid oxidase and urate oxidase are undetectable in HC hepatoma but catalase is as active as in the liver. Hepatomas 7316A and 7794B exhibit high D-amino acid oxidase, urate oxidase and catalase activities. The first two enzymes are also well active in hepatoma 5123A while catalase reaches only 15% of the activity in the rat liver. Hepatoma 7794A is characterized by a high catalase activity; D-amino acid oxidase is weakly active and urate oxidase is not measurable.

*Latency of catalase*

The rat-liver catalase is characterized by its latency in the homogenates, probably because $H_2O_2$ diffusion through the peroxisome membrane is the rate-limiting step in the reaction [18]. This latency disappears if the granule membrane is disrupted by adding a detergent in the incubation medium. Table 2 records the activity of catalase in the tumor homogenates, measured in the absence and in the presence of non ionic detergent Triton X-100. The results have been obtained by a single sample determination for each tumor; they only give a first information about the possible latency of cata-

*Table* 2. *Effect of Triton X-*100 *on catalase activity of hepatomas. The assays were performed on homogenate of the tumor* (a) *without and* (b) *with Triton X-*100 *in the incubation medium. The results have been obtained by a single sample determination for each tumor*

| Hepatoma | Catalase activity Unit/mg protein (a) | (b) |
|---|---|---|
| HC | 0·12 | 0·20 |
| 7316A | 0·03 | 0·17 |
| 5123A | 0·012 | 0·032 |
| 7794B | 0·03 | 0·12 |
| 7794A | 0·28 | 0·28 |

*Table 3. Intracellular distribution of enzymes. Absolute values are given in mg/g for proteins and in unit/g fresh weight of hepatoma for enzymes. The results are given as means ±S.D. E, cytoplasmic extract; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant*

| Enzyme | No. of expts. | Absolute values E+N | Percentage values | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | E+N | N | M | L | P | S | Recovery |
| Hepatoma HC | | | | | | | | | |
| Proteins | 3 | 143±9 | 100 | 29·8±0·7 | 14·8±0·8 | 2·9±0·1 | 17·3±0·6 | 37·1±3·4 | 101·9±3·3 |
| Catalase | 3 | 31·0±0·6 | 100 | 13·5±4·4 | 25·9±2·3 | 19·5±1·0 | 5·3±0·6 | 35·9±8·9 | 100·1±3·9 |
| Hepatoma 7316A | | | | | | | | | |
| Proteins | 3 | 184±7 | 100 | 29·1±2·4 | 21·8±2·6 | 4·0±0·8 | 13·4±0·4 | 30·3±0·9 | 98·6±1·9 |
| Catalase | 3 | 34·8±0·7 | 100 | 14·1±1·4 | 30·5±6·2 | 38·5±3·8 | 4·7±0·8 | 11·9±1·2 | 99·7±10·6 |
| D-amino acid oxidase | 3 | 0·97±0·16 | 100 | 13·8±3·0 | 49·5±1·4 | 43·6±1·4 | 4·8±1·9 | 3·7±1·6 | 115·4±3·9 |
| Urate oxidase | 3 | 1·71±0·18 | 100 | 16·5±2·7 | 30·2±0·4 | 32·6±6·8 | 6·1±1·0 | — | 85·4±3·0 |
| Hepatoma 5123A | | | | | | | | | |
| Proteins | 4 | 150±8 | 100 | 30·5±2·9 | 15·3±0·7 | 5·0±0·4 | 12·4±1·8 | 35·1±1·3 | 98·3±0·7 |
| Catalase | 4 | 4·2±0·8 | 100 | 23·6±4·2 | 25·1±1·8 | 33·6±5·8 | 6·3±2·3 | 13·9±5·1 | 102·5±2·3 |
| D-amino acid oxidase | 4 | 0·30±0·03 | 100 | 16·2±0·7 | 34·7±8·0 | 36·6±6·5 | 7·0±1·5 | 8·9±5·0 | 103·4±7·0 |
| Urate oxidase | 4 | 1·960±0·49 | 100 | 21·2±5·0 | 27·1±4·5 | 36·7±2·6 | 8·1±2·1 | — | 93·1±7·7 |
| Hepatoma 7794B | | | | | | | | | |
| Proteins | 4 | 191±8 | 100 | 38·5±2·2 | 16·3±1·7 | 2·9±0·3 | 10·2±1·2 | 30·6±4·2 | 98·5±2·1 |
| Catalase | 4 | 18·4±4·8 | 100 | 24·1±3·1 | 39·9±4·7 | 24·1±7·0 | 2·9±1·1 | 15·7±6·0 | 106·7±4·6 |
| D-amino acid oxidase | 4 | 0·54±0·19 | 100 | 18·6±3·8 | 51·2±10·2 | 31·5±10·2 | 2·7±1·4 | 7·5±6·4 | 111·5±24·4 |
| Urate oxidase | 4 | 2·55±0·68 | 100 | 24·1±7·2 | 38·4±5·0 | 21·4±2·5 | 2·3±1·8 | — | 86·2±8·9 |
| Hepatoma 7794A | | | | | | | | | |
| Proteins | 4 | 169±8 | 100 | 28·0±2·5 | 13·8±2·2 | 4·5±0·7 | 12·8±1·1 | 38·0±0·4 | 97·1±2·3 |
| Catalase | 4 | 42·4±8·3 | 100 | 3·4±0·6 | 2·0±0·5 | 0·3±0·1 | 3·3±0·4 | 96·2±4·8 | 105·2±5·6 |
| D-amino acid oxidase | 3 | 0·034±0·009 | 100 | 18·6±5·8 | 8·4±2·4 | 4·7±1·7 | 13·0±3·4 | 62·4±6·3 | 107·2±2·2 |

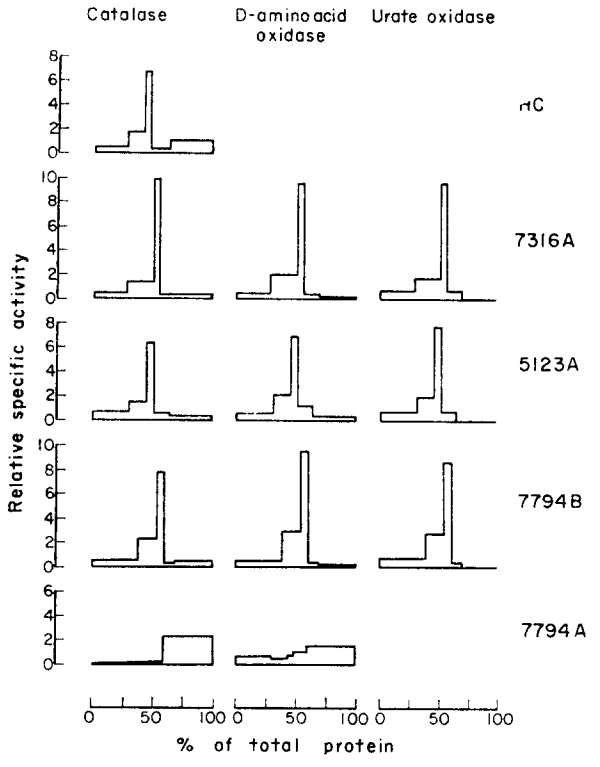


*Fig. 1. Distribution patterns of catalase, D-amino acid oxidase and urate oxidase.* Ordinate: *mean relative specific activity of fractions (percentage of total recovered activity/percentage of total recovered proteins).* Abscissa: *relative protein content of fractions (cumulatively from left to right).*

lase in the homogenate and should not be used for comparing the effect of Triton X-100 from one tumor line to another. As one can see, the catalase activity of hepatomas HC, 7794B, 7316A and 5123A is increased by the addition of the detergent; this increase is sufficiently high to be significant even for a single determination. Conversely, Triton X-100 seems not to affect the catalase of hepatoma 7794A.

*Differential centrifugation*

The mean values for the distributions and the corrected distribution patterns are listed in Table 3 and Fig. 1 as proposed by de Duve *et al.* [16]. In hepatomas 7794B, 7316A, 5123A and HC, catalase exhibits a similar distribution: most of the enzyme is recovered in the mitochondrial fraction and exhibits a peak of specific activity in the light mitochondrial fraction L. However, a relatively high proportion of the activity is present in the soluble fraction of the HC hepatoma homogenate; this situation is to be correlated with the high free activity of the enzyme in the tumor homogenate (Table 2). The catalase distribution of hepatoma 7794A is quite different; 95% of the enzyme is non sedimentable and appears entirely in a free form in the homogenate.

D-amino acid oxidase and urate oxidase distributions in hepatomas 7794B, 5123A and 7316A are comparable to that found for catalase. On the other hand, D-amino acid oxidase, weakly active in hepatoma 7794A, is mainly found in the soluble fraction but to a lesser extent than catalase.

*Isopycnic centrifugation*

In one of the two kinds of experiments performed, the density gradient was established with pure aqueous sucrose. In the other, a macromolecular substance, glycogen, was used to establish the density gradient with a sucrose solution of known molality as the solvent. In such gradients, the density of the particles can be measured in more than one concentration of sucrose [2, 19]. Experiments were usually performed in glycogen gradients extending from 0% to 20% with 0·264, 0·561, 0·988 and 1·470 molal sucrose in water as the solvent. As shown by our differential centrifugation experiments, catalase, D-amino acid oxidase and urate oxidase are mostly found in the total mitochondrial fraction (M+L) except in hepatoma 7794A.

Therefore, in the gradient experiments, our granules were always prepared using a total mitochondrial fraction isolated in 0·25M sucrose. The distribution patterns exhibited by the enzymes in the sucrose gradient are illustrated in Fig. 2. A peak of the catalase activity is observed in the lightest regions of the gradient; it is particularly significant for the preparations isolated from HC and 7794B hepatomas. The major part of the enzymic activity shows a median equilibrium density about 1·21. The presence of a small amount of catalase in the top regions of the sucrose gradient has also been observed after isopycnic centrifugation of rat liver mitochondrial fractions [2]. As shown by Beaufay *et al.* [2] this phenomenon probably results from the fact that the granules suspended in 0·25M sucrose, initially layered at the top of the gradient penetrate in a high sucrose concentration region during the centrifugation and allow a leakage of catalase from the structures with which it was associated. Therefore, the results would indicate that the particles bearing catalase of hepatoma HC and 7794B loose the enzyme more easily than those of hepatoma 5123A and 7316A.

The distribution of D-amino acid oxidase parallels that of catalase; urate oxidase exhibits a similar distribution curve, however no activity is observed in the top regions of the gradient; these results are in accordance with those obtained on rat liver preparations show-

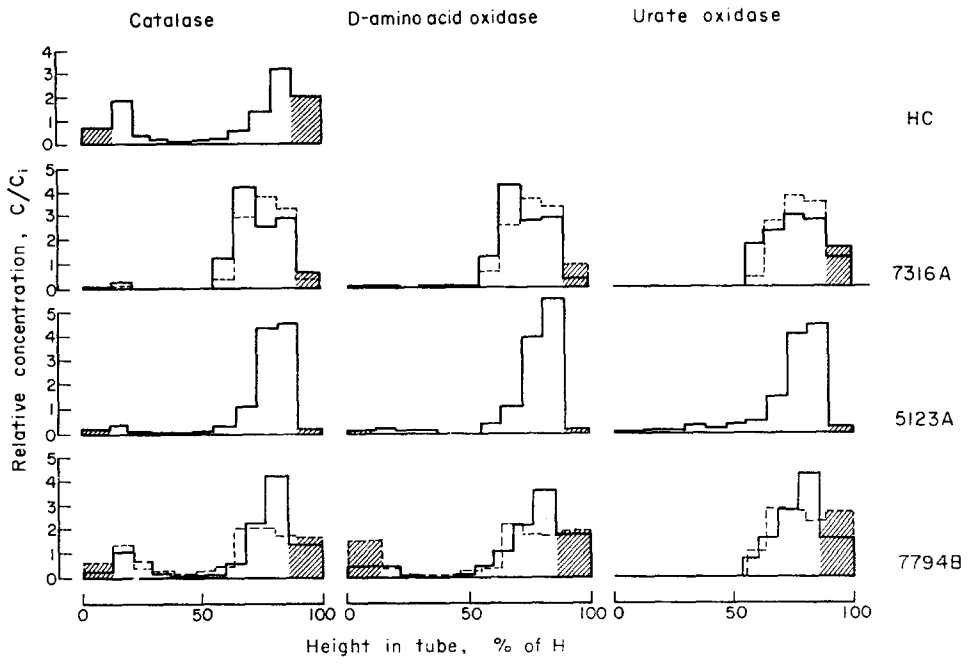


*Fig. 2. Density equilibration of particle-bound enzymes after centrifugation (for 2·5 h at 39·000 rev/min in head SW39 of the Spinco model L-HV preparative ultracentrifuge) of a mitochondrial fraction of hepatoma, through a 0·776 to 3·419 molal sucrose gradient in water. The particles were initially layered on the top of the gradient.* Abscissa: *percentage of the height of the liquid column in the tube (H)*; ordinate: *relative concentration, i.e. ratio of the observed activity (c) to that which would have been observed if the enzyme had been homogeneously distributed throughout the whole gradient* ($c_i$). *Filled blocks* (▨) *are used for the top and bottom subfractions to indicate that they include material falling beyond the limits of the gradient. Two experiments were performed for 7316A and 7794B hepatomas* (—, – – –). *Recoveries are given in Table 5.*

*Table 4. Complementary data on experiments of Fig. 2. Values given refer to the sum of the activities of all the fractions of the gradient expressed in percentages of the total activity included in the gradient*

| Enzyme | HC | 7316A | 5123A | 7794B |
|---|---|---|---|---|
| Catalase | 99 | 122 | 97·1 | 93·4 |
| | | 92 | | 99·3 |
| D-amino acid | — | 94·3 | 94·0 | 88·1 |
| oxidase | | 105·0 | | 98·3 |
| Urate oxidase | — | 110·9 | 88·1 | 105·7 |
| | | 102·0 | | 95·8 |

ing a very tight binding of the enzyme to the particle [2].

In order not to overload the presentation, instead of giving each distribution curve obtained in the glycogen gradients, only the median equilibrium densities are recorded in Table 5. The equilibrium densities are very similar for catalase, D-amino acid oxidase and urate oxidase in each hepatoma and they increase as a function of the density of the sucrose solution in which the particles are suspended.

## DISCUSSION

Studying the behaviour of the enzymes in the appropriate centrifugation systems, it is possible to obtain information about several properties such as density, size, permeability of the subcellular structures with which the enzymes are associated.

In the hepatomas HC, 7794B, 7316A and 5123A, catalase exhibits the phenomenon of latency; most of it is sedimentable and its distribution after differential centrifugation is comparable to that observed by Baudhuin and coworkers for the rat-liver enzyme [1]. These observations suggest that the catalase of the hepatomas examined is associated with particles analogous to those that bind the rat-liver catalase. The behaviour of the enzyme after isopycnic centrifugation strongly argues in favor of this opinion. As shown by Beaufay and Berthet [19], the density of the catalase bearing structures in the rat liver increases linearly in function of the density of the sucrose solution in which they are suspended. According to the authors, these findings may be explained by supposing that the particles bearing catalase do not exhibit an osmotic behaviour: they may be considered like organelles made of a hydrated matrix and a water compartment

*Table 5. Median equilibrium densities* (a) *and recoveries* (b) *of enzymes in glycogen gradients. The density of the sucrose solution is that of the solution in which glycogen is dissolved*

| | Density of the sucrose solution | Catalase | | D-amino acid oxidase | | Urate oxidase | |
|---|---|---|---|---|---|---|---|
| | | (a) | (b) | (a) | (b) | (a) | (b) |
| Hepatoma HC | 1·034 | 1·072 | 101·6% | | | | |
| | 1·068 | 1·101 | 87·0% | | | | |
| | 1·110 | 1·135 | 86·5% | | | | |
| | 1·150 | 1·165 | 86·8% | | | | |
| Hepatoma 7316A | 1·034 | 1·076 | 100·2% | 1·079 | 85·4% | 1·078 | 86·8% |
| | 1·068 | 1·103 | 97·3% | 1·103 | 118·2% | 1·102 | 88·0% |
| | 1·110 | 1·135 | 84·5% | 1·134 | 108·9% | 1·135 | 87·0% |
| | 1·150 | 1·163 | 89·5% | 1·162 | 84·1% | 1·160 | 76·9% |
| Hepatoma 5123A | 1·034 | 1·082 | 89·0% | 1·081 | 108·2% | 1·082 | 121·1% |
| | 1·068 | 1·105 | 86·7% | 1·106 | 103·7% | 1·108 | 99·8% |
| | 1·110 | 1·136 | 93·8% | 1·135 | 99·4% | 1·136 | 134·0% |
| | 1·150 | 1·166 | 85·2% | 1·166 | 85·7% | 1·167 | 114·0% |
| Hepatoma 7794B | 1·034 | 1·077 | 100·6% | 1·076 | 80·7% | 1·079 | 86·0% |
| | 1·068 | 1·109 | 88·9% | 1·104 | 114·6% | 1·114 | 125·9% |
| | 1·110 | 1·133 | 82·5% | 1·132 | 69·0% | 1·134 | 81·0% |
| | 1·150 | 1·162 | 97·5% | 1·163 | 69·3% | 1·162 | 98·6% |

freely accessible to sucrose (sucrose space). In this case the following relationship exists between the density of the particle ($\rho_p$) and the density of the sucrose medium:

$$\rho_p = \frac{\rho_a + \rho_m \beta}{1 + \beta} \qquad \text{(a)}$$

in which $\rho_a$ is the density of the granule matrix which includes all the solid components of the particles and their hydration water, $\rho_m$ is the density of the sucrose solution and $\beta$ the fraction of the volume of the particle freely accessible to sucrose.

An attempt was made to relate the median equilibrium densities of the particles bearing catalase in the four hepatomas to the parameters $\beta$ and $\rho_a$ of the equation (a); the most probable values for these parameters with respect to the experimental data were calculated by the method of least squares. The results are listed in Table 6 and the graphs which fit the equation with these values are drawn in Fig. 3. The data for catalase show that the enzyme in hepatomas HC, 7794B, 5123A, and 7316A is located in particles similar to the ones bearing catalase in the rat liver. As shown in Table 6 the main difference between hepatoma and liver granules consists in the density of the hydrated matrix being significantly lower for the tumor granules.

The same comments may be made for D-amino acid oxidase and urate oxidase in the hepatomas 7316A, 7794B and 5123A; these enzymes seem to be linked to similar structures to those bearing catalase. Nevertheless there is a striking difference in the enzymatic equipment of the organelles in hepatoma HC

*Table 6. Most probable values for the parameters* $\beta$ *and* $\rho_a$ *of particles bearing enzymes.* $\beta$ *is given in* $cm^3.cm^{-3}$ *of hydrated matrix,* $\rho_a$ *in* $g.cm^{-3}$. *The values for the liver enzymes are those reported by Beaufay and Berthet* [19]

| Enzyme | HC | | 7316A | | 5123A | | 7794B | | Liver | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $\beta$ | $\rho_a$ | $\beta$ | $\rho_a$ | $\beta$ | $\rho_a$ | $\beta$ | $\rho_a$ | $\beta$ | $\rho_a$ |
| Catalase | 3·929 | 1·228 | 2·970 | 1·205 | 2·738 | 1·211 | 3·066 | 1·217 | 2·55 | 1·231 |
| D-amino acid oxidase | — | — | 2·844 | 1·203 | 2·823 | 1·213 | 3·400 | 1·218 | 2·40 | 1·224 |
| Urate oxidase | — | — | 3·018 | 1·207 | 2·690 | 1·211 | 2·550 | 1·208 | 2·58 | 1·248 |

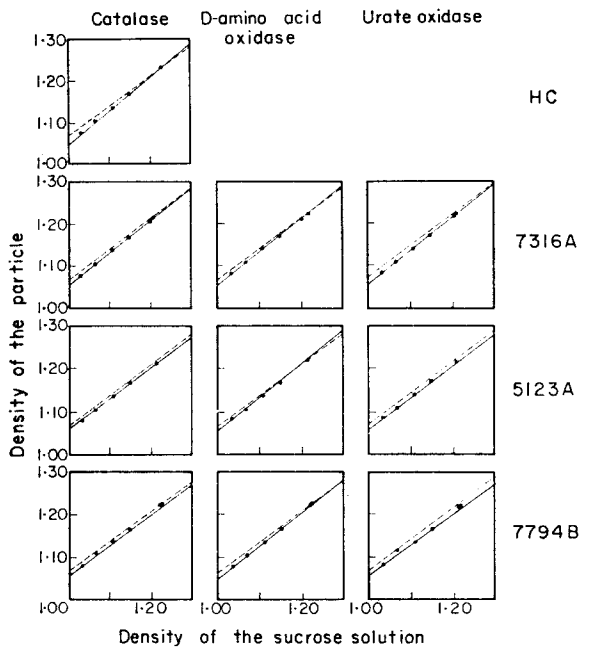


*Fig. 3. Median densities of the particles bearing catalase, D-amino acid oxidase and urate oxidase as a function of the density of the sucrose solution. The values were obtained by estimating the median equilibrium density in glycogen gradients (see Table 4) and for the last point in sucrose gradient. The most probable values of the parameters ß and $\rho_d$ (see text) were calculated by a method of least squares; they are listed in Table 5. The dotted line shows the behaviour of these rat liver enzymes according to Beaufay and Berthet* [19].

and in the rat liver: neither D-amino acid oxidase nor urate oxidase are measurable in that tumor; recently we were unable to detect an L-lactic acid oxidase in that hepatoma. Thus, far, catalase appears to be the sole enzyme present in the peroxisomes of HC hepatoma.

A problem arises for hepatoma 7794A. In the tumor and in the liver, catalase has a similar activity; nevertheless, in the former, the enzyme is found in a soluble form and a high proportion of the soluble D-amino acid oxidase (which is poorly active) is also present. In the rat liver, a relatively high percentage of catalase and D-amino acid oxidase is recovered in the soluble fraction of the homogenate. According to de Duve and Baudhuin [18] the soluble enzymes have their origin in a destruction of peroxisomes by the homogenization procedure. However, the authors do not totally exclude that a part of catalase and D-amino acid oxidase exists in a soluble form inside the cell.

Taking these considerations into account, our results can be explained in two ways: either catalase and D-amino acid oxidase have been solubilized during the preparation of the homogenate which supposes an extreme fragility of the peroxisomes of the hepatoma 7794A; or there are very few peroxisomes in the tumor and we mainly find catalase and D-amino acid oxidase in a free form in the neoplastic cell. In the latter case, the hepatoma 7794A would be endowed with most of the catalase activity in a soluble form. Further aspects of this problem are currently under investigation.

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## SUMMARY

(1) *The activities of catalase, D-amino acid oxidase and urate oxidase have been measured in the hepatoma HC and four Morris hepatomas and compared with those found in the rat liver.* (2) *Catalase shows the phenomenon of latency in homogenates of the hepatomas 7316A, 7794B, 5123A and HC; its activity is very increased by the presence of Triton X-100 in the incubation medium. The detergent has no effect on catalase of the hepatoma 7794A.* (3) *The intracellular distribution of these enzymes has been investigated according to the centrifugation scheme of de Duve and coworkers* [16]. *In the hepatomas 7316A, 7794B, 5123A and HC, the enzymes are mainly recovered in the mitochondrial fractions and exhibit a peak of specific activity in the light one. In the hepatoma 7794A, catalase and D-amino acid oxidase are chiefly present in the soluble fraction.*

(4) *The total mitochondrial fractions of the hepatomas 7316A, 7794B, 5123A and HC have been analysed by isopycnic centrifugation in a sucrose gradient and in gradients made of glycogen dissolved in aqueous sucrose of different concentrations. Catalase, D-amino acid oxidase and urate oxidase of the hepatomas 7316A, 7794B and 5123A exhibit similar distribution patterns. The densities of the particles bearing the enzymes linearly increase as a function of the sucrose concentration of the medium. A similar phenomenon is observed for catalase of hepatoma HC.*

(5) *These results are discussed with respect to the presence of peroxisomes in the hepatomas and to their properties.*

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# Letter to the Editor

## Life Cycle of Lymphoid Cells *in vitro*

H. SMITH, D. MURRAY and M. GÖKCEN
*Life Sciences Foundation, Minneapolis, Minnesota, USA*

### INTRODUCTION

THERE are few reports on the life cycle of lymphoid cells *in vitro*. This communication describes an investigation of the generation times and life cycle of lymphoid cells growing in short-term and in long-term cultures, using pulse-labeling and autoradiographical techniques.

### MATERIAL AND METHODS

For short-term cultures blood was drawn from two normal donors, lightly centrifuged and the buffy coat cells grown in McCoy's 5a Medium* containing 30% fetal calf serum and antibiotics, to which heparin and 0·6 ml phytohemagglutinin P† (PHA) per 100 ml medium were added. Two long-term lymphoid cell cultures were also used. One was a cell line established from lymph node cells of a patient with breast cancer‡, and maintained in continuous culture for over six months. The other cell line was established from the lymph node cells of a patient with Burkitt's lymphoma (JiJoye strain) and maintained in culture for several years.§ The cell lines were grown in McCoy's 5a Medium with 30% fetal calf serum and antibiotics added.

Examination of cultures stained with Wright–Giemsa showed them to possess morphological features typical of lymphoid cells with cells ranging in size from small, medium to large (Fig. 1). Chromosomal karyotyping of the cell cultures showed each to have the classical diploid human karyotype. Occasional tetraploid metaphase spreads were also seen but no consistent chromosomal abnormalities were noted.

The generation time of the lymphoid cells was estimated by pulse-labeling with tritiated thymidine ($H^3$-TdR) followed by autoradiography of chromosome preparation made at regular intervals after $H^3$-TdR labeling. Two long-term lymphoid-cell cultures and two 72 hr PHA stimulated lymphoid cultures from normal donors were used. For pulse-labeling the cells were incubated in medium containing $H^3$-RdR* (1 μC/ml, specific activity 15·8 curies/millimole) for 15 min and then 100 fold excess cold thymidine was added to minimize further labeling. The cells were concentrated by centrifugation and resuspended in fresh medium containing excess cold thymidine thus reducing the effective concentration of any remaining $H^3$-TdR to a negligible amount. They were divided into 24 equal aliquots and incubated at 37°C. Colchicine was added to successive tubes at regular intervals and after one-hour incubation with colchicine-chromosome preparations were made from each tube on microscope slides. The slides were dipped in Nuclear Track Emulsion Type NTB-2† diluted 1 : 1 with distilled water and stored at 4°C for two weeks. They were developed, stained with Wright–Giemsa and examined to determine the number of developed grains

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*Grand Island Biological Co., Grand Island, N.Y. 14072.
†Difco Laboratories, Detroit 1, Michigan.
‡Established in our laboratories.
§Obtained from Grand Island Biological Co., Grand Island, N.Y. 14072.

*New England Nuclear Corp., Boston, Mass. 02118.
†Eastman-Kodak, Rochester, N.Y.

*Table* 1. *Life cycle of lymphoid cells* in vitro

| Authors | Culture conditions | Phases of life cycle in hr $G_1$ | S | $G_2+M$ | Generation time in hr |
|---|---|---|---|---|---|
| Smith, *et al.* 1969 (present report) | PHA stimulation | 1·2 | 10·0 | 3·8 | 15·0 |
| | PHA stimulation | <1 | 10·2 | 3·8 | 15·1 |
| | Continuous culture of lymphoid cells from breast cancer patient | 3·8 | 9·8 | 3·4 | 17·0 |
| | Continuous culture of lymphoid cells from Burkitt lymphoma patient | 3·5 | 9·1 | 8·9 | 21·5 |
| Cave, 1966 [1] | PHA stimulation | 4·6 | 9·6 | 3·5 | 17·7 |
| Takagi and Sandberg, 1968 [2] | PHA stimulation | <2 | 7·6 | 2·9 | 12·5 |
| | PHA stimulation | <1 | 8·3 | 2·6 | 11·5 |

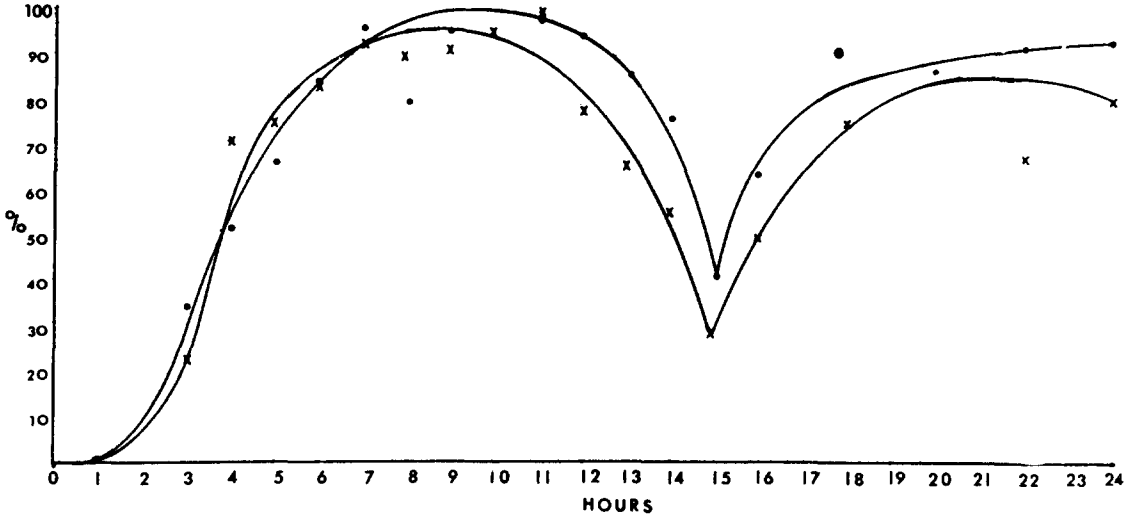


*Fig.* 2. ●—●; X—X, *PHA stimulated lymphoid cultures from two normal donors. Percentage of labeled metaphases compared to total number of metaphases at successive hourly intervals after labeling.*

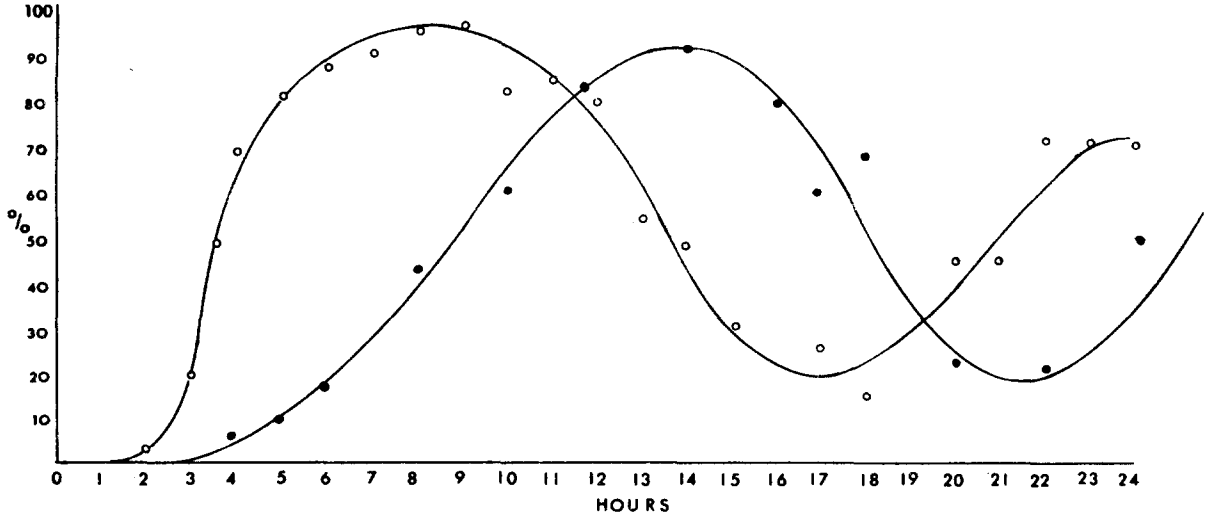


*Fig.* 3. ○—○ *continuous culture of lymphoid cells derived from patient with breast cancer.* ●—● *continuous culture of lymphoid cells from patient with Burkitt lymhoma. Percentage of labeled metaphases compared to total number of metaphases at successive* 1*-hr and* 2*-hr intervals after labeling, respectively.*

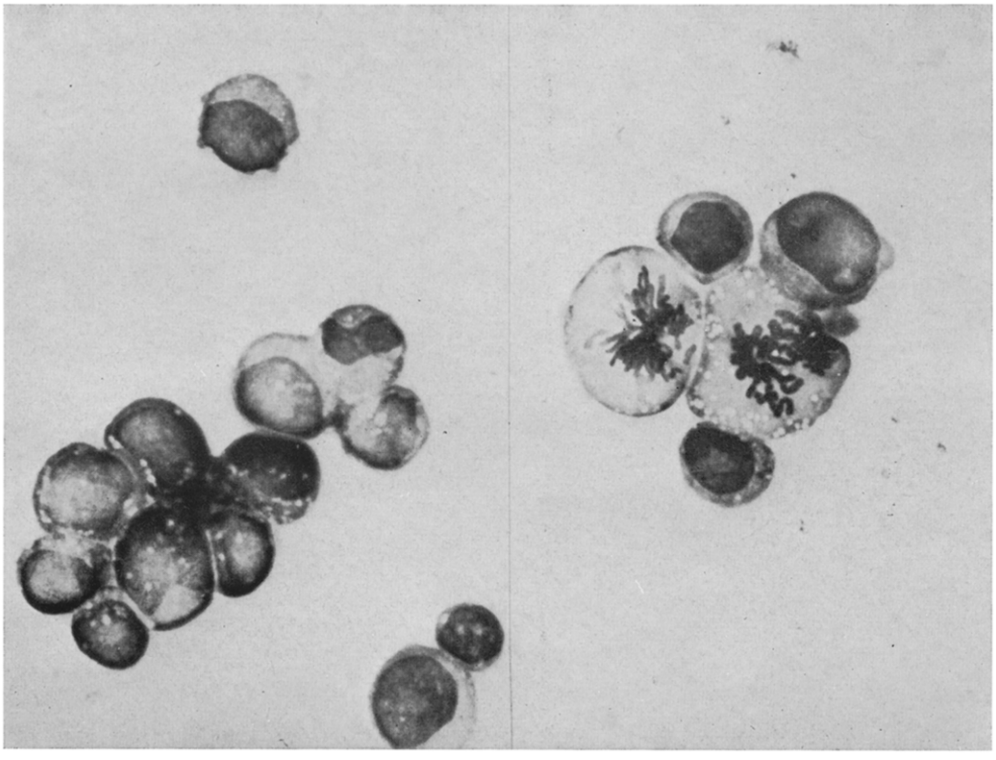

*Fig.* 1. *Lymphoid cells in culture. Wright–Giemsa stain.* × 1000.

(to face p. 270)

over the chromosome spreads. The generation time and life cycle of the cultured cells was estimated by graphing the number of labeled metaphases compared to the total number of metaphases with time (Figs. 2 and 3). A minimum of 50 well spread metaphases were counted for each point. From the graph the total generation time and phases in the life cycle of the cells could be estimated. The results are shown in Table 1 along with recent findings of other investigators for comparison. We are unable to explain the differences observed by different investigators.

In our experiments we found the S phase to be fairly similar in short-term and long-term cultures. PHA stimulated cells had S phases of 10·0 hr and 10·2 hr while the long-term cultures had S phases of 9·8 hr and 9·1 hr. The $G_1$ phase of 3·8 and 3·5 hr of long-term cultured cells was clearly different from the compressed $G_1$ phase of 1·2 hr and $<1$ hour shown by PHA stimulated cells. As PHA stimulated cells appear to have a limited life span *in vitro*, a minimum $G_1$ phase of 3–4 hr appears to be necessary for continued proliferation in culture. The $G_2+M$ phase was similar in lymphoid cells from the breast cancer patient and in PHA stimulated cells, being 3·4 hr and 3·8 hr respectively. The culture derived from Burkitt lymphoma patient, however, showed a markedly longer $G_2+M$ phase of 8·9 hr, which could possibly be associated with the presumed malignant nature of these cells.

The differences in the various phases of the life cycle of the cultures are reflected in their generation times, short-term PHA stimulated cultures had generation times of 15·0 hr and 15·1 hr. Lymphoid cells from the breast-cancer patient had a generation time of 17·0 hr and the culture of Burkitt lymphoma cells had a generation time of 21·5 hr.

Cells in culture especially after prolonged culturing probably constitute a fairly select homogeneous population of cells. Radioactive tagging of lymphoid cells *in vivo* has shown that lymphoid cells are a heterogenous population with some cells being short-lived while others may persist for prolonged periods of time [1, 2]. Cells taking part in an immunological response may show generation times of 8–12 hr [3–5].

As the long-term cultures were derived from tissues which were composed of a heterogeneous population of lymphoid cells, their extended generation times show that other factors apart from the rate of cell proliferation must play a part in the eventual establishment of a cell line.

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# Mammary-Tumor Virus Activity in Brain and Liver of GR Strain Mice

P. BENTVELZEN and J. H. DAAMS

*Radiobiological Institute TNO, Rijswijk Z.H., The Netherlands; and The Netherlands Cancer Institute, Amsterdam*

## INTRODUCTION

THE GR mouse strain harbors a mammary-tumor virus variant (MTV-P), which is characterized by the induction of pregnancy-dependent tumors [1], called plaques [2]. In contrast to the standard mammary-tumor virus (MTV-S), this virus is — in the GR strain — transmitted not only by means of the milk but also by the gametes [1, 3, 4, 5]. If the virus is introduced into other mouse strains, however, only transmission through the milk occurs [1, 4]. It has been postulated that MTV-P can be transmitted as a genetic factor of the GR host [1, 4, 6–8].

Because of the renewed interest in the distribution of MTV in the mouse [9–11], we started an investigation of this problem in the GR mouse strain. In indirect immunofluorescence studies with polyvalent rabbit antisera to purified MTV-S, viral antigens were detected in mammary gland, epididymis and lymphoid organs of mice from several strains [12]. The GR strain proved to be unique in that some antisera could detect an MTV antigen also in livers and brains from this strain. The present report deals with the question whether this presence of viral antigens in the liver and brain is associated with biological activity.

## MATERIAL AND METHODS

The following inbred mouse strains were used:

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(The standardized nomenclature for inbred mouse strains [13] is employed)
C3H/HeA, which carries the virulent MTV-S,
GRSI/A, which carries the moderately virulent MTV-P,
BALB/cAnDe, which is a low-mammary-tumor-strain but which is highly susceptible to both MTV-S and MTV-P,
BALB/cfC3H/A, which carries MTV-S,
BALB/cfGR/A, which carries MTV-P.

For mammary-tumor induction, two 4-week old female mice were caged with one 2-month old hybrid male, which was free from either MTV-P or MTV-S. The cages were inspected daily for the presence of newborn litters, which were immediately removed. Twice a week the females were examined for the presence of tumors. Tumor-bearing females were separated and the response of the tumor on parturition was observed. When the females were 1 year old most experiments were terminated.

For the collection of blood 2-month old female mice were decapitated after a light ether narcosis. One tenth of a ml of blood per mouse was then injected into 4-week old BALB/c females, which then were subjected to forced breeding.

Livers were perfused with a solution containing 0·04 M sodium citrate (pH 7·2)+ 0·15 M sucrose. Cell-free homogenates of perfused livers and brains were prepared according to the technique of Timmermans *et al.* [14]. One g of tissue was homogenized in 10 ml of a solution of 0·6% NaCl+0·04% bovine serum albumin+0·02 M McIlvaine phosphate–

citrate buffer and then centrifuged at 1500 **g** for 5 min. The sediment was homogenized again and then recentrifuged. The two supernatants were combined and then spun at 10,000 **g** for 15 min. The cell-free extract was taken from the upper half of the supernatant and injected intraperitoneally into 4-week old BALB/c mice (0·5 ml per mouse). The mice were subjected to forced breeding as described above.

## RESULTS

The tumor incidences at 12 months of age in the different mouse strains used in this investigation are reported in Table 1. The BALB/c strain gets only 1% tumors at that age. A small group of surviving mice was kept much longer under observation; a final incidence of 30% (6 out of 20) was obtained with an average tumor age of 18 months. This is in agreement with data of Deringer [15], from whom this line was acquired.

All BALB/cfC3H mice, which carry MTV-S get a tumor before 1 year of age, just as in the C3H mouse strain. In the BALB/cfGR strain, however, not all mice developed such a lesion before that age. The final incidence at 2 years of age is 90% (46 out of 51). It seems that MTV-P is somewhat less virulent than MTV-S. The 100% incidence of those lesions in the GR strain indicates the greater susceptibility of this strain to the oncogenic action of MTV-P. All tumors in the GR strain are pregnancy-dependent whereas only a few tumors induced by MTV-S show this characteristic. In the BALB/cfGR the majority of the tumors are pregnancy-dependent also. This means that pregnancy-dependency is controlled by the virus, although host factors seem to play a minor but definite role.

In Table 2 are reported tumor incidences in groups of BALB/c mice, which were injected with blood or with liver or brain homogenates of mice from other strains. Blood from BALB/cfC3H and BALB/cfGR donors induced many tumors whereas blood from C3H and GR mice induced only 25% and no tumors respectively. This difference most likely can be explained by the dependence of bloodborne MTV on the H-2 transplantation antigens [16].

Inoculations with GR liver and brain extracts induced 35 and 29% of tumors respectively, whereas the extracts from other mouse strains were negative. The positive results with the GR extracts are not due to contaminating blood, not only because we worked with perfused livers but also in view of the negative results with GR blood and the extracts from brains and livers of the other mouse strains.

Three of the tumors induced by the brain extract and two of those induced by the liver extract were pregnancy-dependent. This indicates still more that the tumors in these groups are not due to activation of a latent MTV in

*Table* 1. *Mammary-tumor incidences at one year of age in forced bred females of several inbred mouse strains*

| Strain | No. of mice | No. with tumors | % | No. of pregnancy-dependent tumors | % |
|---|---|---|---|---|---|
| BALB/c | 81 | 1 | 1 | 0 | 0 |
| BALB/cfC3H | 18 | 18 | 100 | 1 | 6 |
| C3H | 24 | 24 | 100 | 2 | 8 |
| BALB/cfGR | 51 | 36 | 71 | 22 | 61 |
| GR | 23 | 23 | 100 | 23 | 100 |

*Table* 2. *Mammary-tumor incidences at one year of age in forced bred BALB/c females inoculated with materials from other mouse strains*

| Nature of material | Tumor incidences at one year* Donor strains | | | |
|---|---|---|---|---|
| | C3H | BALB/cfC3H | BALB/cfGR | GR |
| Whole blood | 3/12 (25) | 9/10 (90) | 9/12 (75) | 0/22 (0) |
| Cellfree liver extract | 0/18 (0) | 0/27 (0) | 0/33 (0) | 7/20 (35) |
| Cellfree brain extract | 0/24 (0) | 0/22 (0) | 0/15 (0) | 5/17 (29) |

*Reckoner, number of mice with a tumor; denominator, total number of mice. Between brackets percentage.

the BALB/c strain, because pregnancy-dependency of BALB/c tumors has never been observed by us, not even when they appear early due to treatments with urethane and/or X-rays [17]. We therefore conclude that livers and brain of GR mice contain some biologically active form of MTV-P.

## DISCUSSION

The MTV virions called B particles [18] are produced in the mammary gland and in the epididymis [19]. In the latter organ, however, no tumors arise under influence of MTV-S. There are some indications that lymphoid organs, in which MTV antigens are present [12], contain a biologically active form of the virus [20]. In these organs no B particles have been observed so far, but in those of aged mice intracytoplasmic A particles are seen [21] which are thought to be the precursor of B [22]. Nandi [9] and Moore [11] found MTV activity in the erythrocytes, which suggests that the hemopoietic stem cell is the primary site of MTV multiplication. We have never got an indication, however, that MTV induces neoplasms in the blood forming organs. It seems that the different functions of the MTV genome like virion synthesis and neoplastic transformation are strongly influenced by the epigenetic status of the host cell.

On the basis of our immunological data [12] and the present biological findings, we postulate that only MTV-P-RNA and a few (or only one) virus-specific proteins are present in the brains and livers of GR mice. The presence of some form of MTV-P in GR brain and liver versus its absence from the corresponding BALB/cfGR organs is undoubtedly related to the remarkable modes of vertical transmission of this virus in the GR strain. It is certainly compatible with the idea of a genetic transmission of MTV-P in the GR strain [1, 4, 6–8], on the basis of which it can be expected that MTV-P-RNA will be present in every cell, although it need not replicate and function normally.

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## SUMMARY

*Only* 1 *out of* 81 *forced bred BALB/c females got a mammary tumor before* 1 *year of age. Extracts from livers and brains of GR-strain mice induced in forced bred BALB/c mice* 35 *and* 29% *mammary tumors respectively, whereas extracts from these organs of C3H, BALB/cfC3H and BALB/cfGR were inactive.*

*Whole blood of BALB/cfC3H and BALB/cfGR mice induced many mammary tumors in BALB/c mice, whereas blood from C3H mice induced only* 25% *and from GR mice* 0%. *The latter finding excludes the possibility that the positive results with GR liver and brain extracts are due to contaminating blood. It is concluded that these organs contain some biologically active form of the mouse mammary-tumor virus harbored in GR mice. The results are discussed in relation to the hypothesis of a genetical transmission of this virus in the GR strain.*

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# The Influence of Host Factors on Cyclophosphamide Treatment of Moloney Virus-Induced Lymphomas*

SVEN BREMBERG

*Department of Tumor Biology, Karolinska Institutet Medical School, 104 01 Stockholm 60, Sweden*

## INTRODUCTION

IT IS USUALLY impossible to eliminate all tumor cells present in a cancer patient by surgery or radiotherapy. Efforts to destroy the remaining cells by chemotherapy or by stimulation of host immunoreactivity against the tumor seldom succeed but recent attempts in combining these methods have yielded promising results [1]. Moreover, it is currently suggested that the outstanding effect of cytostatic drugs on choriocarcinoma [2, 3] and Burkitt's lymphoma [4, 5] may be due to concomitant immunoreactivity against these tumors. Thus under certain conditions immune mechanisms may not be abolished by these potentially immunodepressive drugs but may increase the therapeutic efficiency of the drug.

In animal systems evidence has been obtained for improvement of cytostatic drug effect by host immunoreactivity. Previous host contact with antigens present on the tumor cells [6] — either in the form of regressed growths [7] or injection of irradiated cells [8] — has been found to increase the therapeutic effectiveness of subsequently administered cytostatic drugs. The growth of Sarcoma 780 has been found to be more effectively inhibited by cytostatic treatment in random bred Swiss mice than in a strain with closely matching histocompatibility antigens [9]. In mice with impaired immunoreactivity due to X-irradiation [10], neonatal thymectomy [11, 12] or cortisone-treatment [10] drug effects were found to be weaker. In these systems, involving allogeneic animals immunoreactivity was directed against both tumor specific and histocompatibility antigens. It is therefore of interest to investigate the situation in a syngeneic system utilising tumors with good tumor specific antigens. Moloney virus induced (MLV) lymphomas were chosen since they carry a tumor specific, virus determined antigen, detectable *in vivo* and *in vitro*. Humoral antibodies reactive with surface antigens of MLV cells can be demonstrated in grafted or immunized mice [13]. Immune reactivity of the individual animal against their tumors can be increased specifically by pretreatment with irradiated or allografted MLV lymphoma cells or Moloney virus containing homogenates [13]. It can be decreased by total body irradiation or even abolished by neonatal virus injection which frequently causes tolerance [14]. In these ways it is possible to obtain a spectrum of strength of the host response. A further variation of the system is provided by the availability of lymphoma cell lines varying in immunosensitivity.

In this study cyclophosphamide treatment was applied to various host–tumor systems in order to obtain an overall view of the problem. On the basis of the results of this survey specific systems could be selected for further study in order to unravel the interaction of the

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various components contributing to successful growth inhibition.

## MATERIAL AND METHODS

*Mice* of both sexes, (A/Sn and CBA), weighing 20–25 g were used. $F_1$ hybrids of the following crosses were also employed: A/Sn × C3H, A/Sn × C57B1, A/Sn × C57L, A/Sn × DBA/2, A.BY × DEA/2, CBA × C3H and C3H × C57B1. All strains were maintained by continuous single line, brother and sister matings in this laboratory.

*Lymphomas* were induced by neonatal inoculation of Moloney virus containing homogenates. Serially transplanted lines were maintained by passage in syngeneic non-irradiated mice. The tumors grew as generalized lymphomas or as subcutaneous or intraperitoneal masses, depending on the line and on the route of inoculation. For transplantation, solid tumors or grossly enlarged lymphoid tissues were removed, pressed through a stainless steel screen and suspended in balanced salt solution (BSS). Lymphoma cells growing in ascitic form (YAC and YAC-IR) were collected by rinsing the peritoneal cavity with BSS. The viability of the cells was assessed by trypan blue exclusion. Only suspensions with more than 50% viable cells were used. The concentration of cells was determined by counting in a Bürker chamber. A predetermined number of viable (unstained) lymphoma cells was suspended in 0·1 ml volumes and inoculated subcutaneously in the right flank of the strain of origin or an $F_1$ hybrid derived from a cross between the original strain and a foreign strain. The day of grafting was taken as day 0.

The lymphomas are designated according to the nomenclature used at this laboratory. The first letter Y refers to the induction by the Moloney virus. The second letter (second and third for four letter designations) indicates the strain of origin according to Table 1. YAC-IR is an immuno-resistant subline of YAC (provided by Dr. E. M. Fenyö [15]). The original YAC line is highly sensitive to the cytotoxic effect of Moloney-specific antibodies *in vitro*, whereas the IR line is almost completely resistant. Sensitivity was not regained after 10 serial passages in non-immune mice [15].

*Immunization.* YHA tumor cells were irradiated with 6000 r (1 mm Al filter, 30 cm distance) and $5 \times 10^6$ of these cells were inoculated subcutaneously into the left flank 30–45 days prior to the experiment. The irradiated cells did not give rise to tumors in any case. Mice treated in this way develop humoral antibodies against MLV lymphomas [13, 16]. The presence of these antibodies was checked in some experiments by the conventional cytotoxic test [15].

*Tolerance.* Mice were inoculated with frozen and thawed MLV material during their first day of life. Four to six weeks later serum was collected and the cytotoxic test performed [15]. Mice with cytotoxic index below 0·10 were designated as tolerant. Such mice usually do not develop humoral antibodies to MLV lymphoma cells. Cell-bound immunity is also depressed since they exhibit no or reduced transplantation resistance to the corresponding lymphoma cells [17, 14].

*Irradiation.* Mice were exposed to whole body irradiation with 400 r (0·5 mm Cu, 41 cm distance) immediately before grafting of a tumor.

*Cyclophosphamide treatment.* On the first day when 50% of the animals were noted to have

*Table* 1. *MVI lymphomas studied*

| Lymphoma designation | Mouse of origin: strain and sex | Transplantation passage used |
|---|---|---|
| YAA | A/Sn♂ | 28 |
| YAB | A/Sn♂ | 51–53 |
| YAC | A/Sn♀ | 162–232 |
| YALB | (A/Sn × C57L)$F_1$♂ | 15–17 |
| YA7C | (A/Sn × C57B1)$F_1$♂ | 20 |
| YBA | CBA♂ | 11–55 |
| YBB | CBA♀ | 18 |
| YBC | CBA♂ | 45–48 |
| YDAG | (A/Sn × DBA/2)$F_1$♂ | 27 |
| YDYA | (DBA/2 × ABY)$F_1$♂ | 11 |
| YHA | C3H♂ | 7–12 |
| YHC | C3H♂ | 13–26 |
| Y7HA | (C3H × C57B1)$F_1$♀ | 22–45 |
| YLD | C57L♀ | 122–128 |

*Table 2. Effect of cyclophosphamide on tumors in pre-irradiated mice*

| Experiment No. | Tumor: Line | Tumor: Passage | Tumor: No. of cells grafted | Recipient mice: Strain | Recipient mice: Sex | Recipient mice: Pretreatment | CPA treatment day No. | Fraction of CPA treated mice with tumors at day of treatment¶ | Median remission time (RT) (days) | Median survival time (days): Controls | Median survival time (days): CPA treated | Increase for CPA treated (IMST) (days) | Fraction of tumor free mice, alive, on day 100: Controls | Fraction of tumor free mice, alive, on day 100: CPA treated |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | YAA | 28 | $10^5$ | (A/Sn × C57Bl)$F_1$♂ | | — | 10† | 3/4 | 16 | 35 | 92 | +57 | 1/4 | 2/4 |
| | | | | | | IRR | 8 | 3/4 | 10 | 24 | 36 | +12 | 0/4 | 0/4 |
| II | YAB | 53 | $10^7$ | (A/Sn × C57L)$F_1$♀ | | — | 7† | 4/4 | 8 | 15 | 30 | +15 | 0/4 | 0/4 |
| | | | | | | IRR | 7 | 4/4 | 8 | 15 | 27 | +12 | 0/4 | 0/4 |
| III | YAC | 163 | $10^6$ | (A/Sn × C57Bl)$F_1$♂ | | — | 7† | 2/4§ | >89 | 16 | 88 | +72 | 0/4 | 2/4 |
| | | | | | | IRR | 7 | 2/3 | 17 | 13 | 37 | +24 | 0/4 | 0/3 |
| IV | YAC | 228 | $10^4$ | (A/Sn × C3H)$F_1$♂ | | — | 9* | 4/4 | 13 | 20 | 33 | +13 | 0/4 | 0/4 |
| | | | | | | IRR | 7 | 3/3 | 11 | 20 | 23 | + 3 | 0/4 | 0/3 |
| V | YAC-IR | 24 | $10^6$ | (A/Sn × C57Bl)$F_1$♀ | | — | 11† | 4/4 | 10 | 10 | 22 | +12 | 0/4 | 0/4 |
| | | | | | | IRR | 11 | 4/4 | 12 | 10 | 20 | +10 | 0/4 | 0/4 |
| VI | YALB | 15 | $10^6$ | (A/Sn × C57L)$F_1$♂ | | — | 11† | 3/4 | >86 | 52 | 100 | >48 | 0/4 | 4/4 |
| | | | | | | IRR | 11 | 3/4 | 27 | 28 | 50 | +22 | 0/4 | 0/4 |
| VII | YA7C | 20 | $10^7$ | (A/Sn × C57Bl)$F_1$♀ | | — | 9† | 4/4 | >88 | 36 | 100 | >64 | 0/4 | 3/4 |
| | | | | | | IRR | 9 | 4/4 | 37 | 23 | 70 | +47 | 0/4 | 1/4 |
| VIII | YBC | 45 | $10^4$ | (CBA × C3H)$F_1$♂ | | — | 8* | 4/4 | 13 | 17 | 27 | +10 | 0/4 | 0/4 |
| | | | | | | IRR | 8 | 2/3 | 10 | 15 | 31 | +16 | 0/4 | 0/3 |
| IX | YDAG | 27 | $10^6$ | (A/Sn × DBA/2)$F_1$♂ | | — | 12‡ | 3/4§ | 9 | 27 | 52 | +25 | 0/4 | 0/4 |
| | | | | | | IRR | 12 | 4/4 | 9 | 18 | 31 | +13 | 0/3 | 0/4 |
| X | YDYA | 11 | $10^4$ | (A.BY × DBA/2)$F_1$♀ | | — | 7‡ | 2/4 | 14 | 28 | 30 | + 2 | 1/4 | 1/4 |
| | | | | | | IRR | 7 | 2/4 | 9 | 20 | 27 | + 7 | 0/4 | 0/4 |
| XI | Y7HA | 45 | $10^7$ | (C3H × C57Bl)$F_1$♂ | | — | 11‡ | 3/4§ | 4 | 17 | 25 | + 8 | 0/4 | 0/4 |
| | | | | | | IRR | 11 | 2/3 | 4 | 23 | 38 | +15 | 0/3 | 0/3 |
| XII | YLD | 128 | $10^5$ | (A/Sn × C57Bl)$F_1$♀♂ | | — | 14† | 2/4 | 5 | 21 | 64 | +43 | 0/4 | 2/4 |
| | | | | | | IRR | 10 | 4/4 | 0 | 14 | 19 | + 5 | 0/4 | 0/4 |

*CPA dose 100 mg/kg body weight.
†CPA dose 150 mg/kg body weight.
‡CPA dose 200 mg/kg body weight.
¶The median tumor diameter ranged 2–8 mm. It was equal for unirradiated and irradiated groups except in § indicated experiments.
§The median tumor diameter of irradiated groups 2–3 mm greater than for unirradiated.

Lymphomas were grafted to unirradiated and whole body irradiated (IRR) (400 r) syngeneic recipients. On the first day when at least 50% of the mice carried palpable tumors each group was divided in two equal subgroups. One was treated with a single dose of cyclophosphamide (CPA), the other served as control.

palpable tumors, two groups were selected and assigned as controls or for CPA treatment. Tumor-bearing mice were equally distributed between the two groups, with a similar range of tumor size in each group. Cyclophosphamide* was given in a single intraperitoneal

*Sendoxan, Pharmacia, Uppsala, Sweden.

injection in a dose roughly corresponding to the $LD_{10}$ for irradiated normal mice of the strain. The drug was dissolved in 0·1 ml saline immediately before use to give a final concentration corresponding to the appropriate CPA dose for a 20 g mouse.

*Evaluation of cyclophosphamide effect.* Most tumors grew subcutaneously at the site of injection. Three diameters at right angles to each other were measured by caliper, twice a week. For animals without localized tumors, the diameters of the inguinal lymph node on the right side was measured. At the start of CPA treatment the median of the mean diameters of the individual tumors was determined. The animals were usually tumor-free 3 to 4 days after CPA treatment. The duration of the remission (remission time, RT) was calculated as the interval between the first day of complete regression and the median time of recurrences.

The increase in median survival time (IMST) was compared between CPA treated and control animals. Experiments in which tumor-free animals died were excluded.

*Cytotoxic test.* This was performed as described previously [15].

## RESULTS

*Effect of cyclophosphamide in pre-irradiated mice* (*Table* 2). In 12 experiments the effect of CPA was studied on tumors grafted to pre-irradiated mice. In experiment I, III, VI, VII and XII the CPA treatment resulted in pronounced remissions including several tumor-free long term survivors. In these five experiments the treatment effect was definitely weaker in the corresponding irradiated groups: the IMST and RT were shorter and the proportion of long term survivors lower. In the remaining experiments the CPA effect was slight (IMST 2–15 days) even in non-irradiated hosts. In the irradiated subgroups even this slight effect was somewhat impaired in 4 experiments (almost the same as for the non-irradiated in two experiments) and only in one experiment (XI) was the effect slightly better in the irradiated group. The decreased immunoreactivity in irradiated mice was verified in experiment VI. Sera from day 30, 35, 42 and 48 were assayed for cytotoxic effect (Fig. 1). Antibodies with titers of 1 : 8–1 : 32 were detected only in the non-irradiated mice.

It seems that pronounced therapeutics effects of CPA were substantially impaired in irradiated (i.e. less immunoreactive) mice.

*Effect of cyclophosphamide in pre-immunized mice* (*Table* 3). The reactivity of the host against the tumor was augmented by pre-immunization in ten experiments. In mice not receiving CPA, pre-immunization prolonged the median survival time by 3–21 days. Irradiation at the time of tumor inoculation (in experiment II, III, IV, VII, VIII and XI) — which does not impair a secondary immune response — was

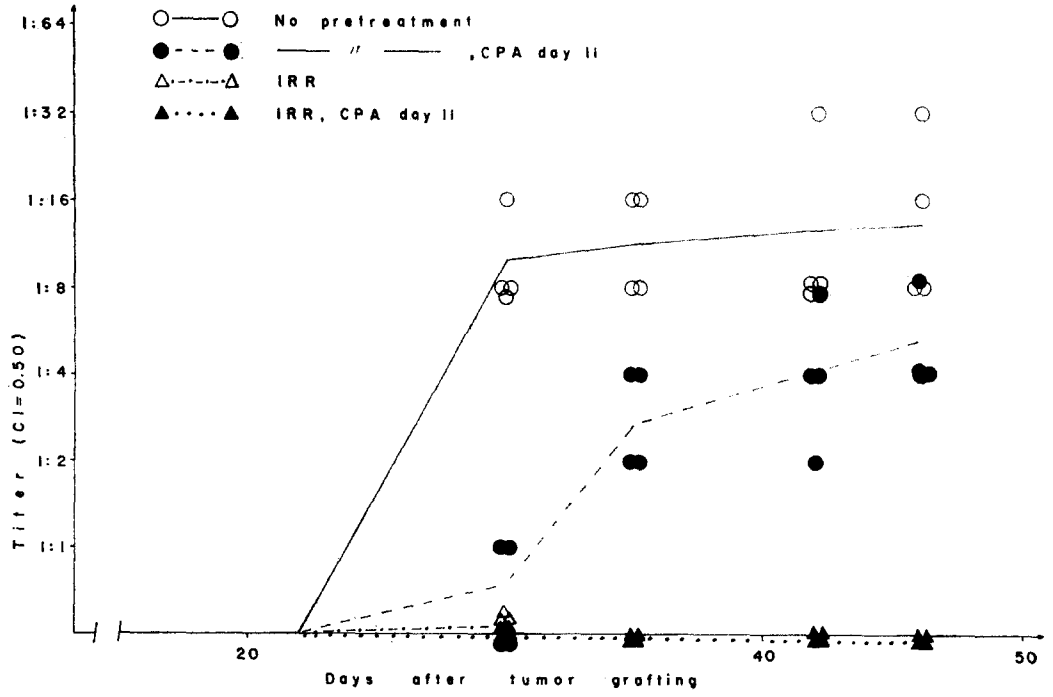


*Fig.* 1. *Development of cytotoxic antibodies in* $(A/Sn \times C57L)F_1$ *mice grafted with YALB lymphoma cells and treated with cyclophosphamide* (*CPA*) *on day* 11. *IRR designated groups irradiated with* 400 *r before tumor grafting* (*Exp. VI, Table* 2).

*Table 3. Effect of cyclophosphamide on tumors in pre-immunized mice*

| Experiment No. | Tumor: Line | Tumor: Passage | Tumor: No. of cells grafted | Recipient mice: Strain | Recipient mice: Sex | Pre-treatment | CPA treatment day No. | Fraction of CPA treated mice with tumors at day of treatment¶ | Median remission time (RT) (days) | Median survival time (days): Controls | Median survival time (days): CPA treated | Increase for CPA treated (IMST) (days) | Fraction of tumor free mice, alive on day 100: Controls | Fraction of tumor free mice, alive on day 100: CPA treated |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | YAB | 53 | $10^7$ | (A/Sn × C57L)$F_1$ | ♀ | — — | 7‡ | 4/4 | 7 | 15 | 28 | 13 | 0/4 | 0/4 |
| | | | | | | IM — | 11 | 4/4 | 10 | 18 | 36 | +18 | 0/4 | 0/4 |
| II | YAC | 80 | $10^5$ | (A/Sn × C57B1)$F_1$ | ♀ | —IRR | 11* | 3/4§ | 8 | 16 | 31 | +15 | 0/4 | 0/4 |
| | | | | | | IM+ IRR | 20 | 3/4 | 16 | 37 | 51 | +14 | 0/4 | 0/4 |
| III | YALB | 17 | $10^7$ | (A/Sn × C57L)$F_1$ | ♂ | —IRR | 12† | 2/3 | 0 | 22 | 44 | +22 | 0/4 | 0/3 |
| | | | | | | IM+ IRR | 12 | 2/3 | >84 | 34 | >100 | >66 | 0/3 | 2/3 |
| IV | YA7C | 20 | $10^7$ | (A/Sn × C57B1)$F_1$ | ♀ | —IRR | 9† | 4/4 | 37 | 23 | 70 | +47 | 0/3 | 1/4 |
| | | | | | | IM+ IRR | 9 | 4/4 | 33 | 26 | 63 | +37 | 0/4 | 0/4 |
| V | YBA | 15 | $10^7$ | CBA | ♀ | — — | 12‡ | 3/3 | >83 | 25 | >100 | >75 | 0/3 | 2/3 |
| | | | | | | IM — | 12 | 3/3 | 32 | 38 | 68 | +30 | 0/3 | 0/3 |
| VI | YBA | 55 | $10^7$ | CBA | ♂ | — — | 7* | 3/3 | 5 | 20 | 26 | + 6 | 0/3 | 0/3 |
| | | | | | | IM — | 9 | 3/4 | 12 | 18 | 35 | +17 | 0/4 | 0/4 |
| VII | YBB | 18 | $10^7$ | CBA | ♂ | —IRR | 11† | 2/3§ | 0 | 21 | 26 | + 5 | 0/2 | 0/3 |
| | | | | | | IM+ IRR | 11 | 3/3 | 10 | 18 | 40 | +22 | 0/4 | 0/3 |
| VIII | YBC | 48 | $10^5$ | CBA | ♀ | —IRR | 13† | 4/4 | 8 | 20 | 28 | + 8 | 0/4 | 0/5 |
| | | | | | | IM+ IRR | 16 | 2/3 | 11 | 28 | 38 | +10 | 0/3 | 1/3 |
| IX | YDAG | 27 | $10^6$ | (A/Sn × DBA/2)$F_1$ | ♂ | —IRR | 12‡ | 4/4 | 0 | 18 | 31 | +13 | 0/3 | 0/4 |
| | | | | | | IM+ IRR | 12 | 3/3 | 18 | 31 | 50 | +19 | 0/3 | 0/3 |
| X | Y7HA | 22 | $10^7$ | (C3H × C57B1)$F_1$ | ♀ | — — | 7‡ | 4/4 | 7 | 22 | 27 | + 5 | 0/4 | 0/4 |
| | | | | | | IM — | 15 | 3/4 | 7 | 25 | 36 | +11 | 0/4 | 0/4 |

*CPA dose 100 mg/kg body weight.
†CPA dose 150 mg/kg body weight.
‡CPA dose 200 mg/kg body weight.
¶The median tumor diameter ranged 2–9 mm. It differed 2 mm or less between immunized and non-immunized groups except in § indicated experiments.
§The median tumor diameter of immunized groups 5 mm greater than for non-immunized.

Lymphomas were grafted to preimmunized (IM) and non-immunized syngeneic recipients. In some experiments both groups were irradiated (IRR) (400 r) before grafting. On the first day when at least 50% of the mice carried palpable tumors each group was divided in two equal subgroups. One was treated with a single dose of cyclophosphamide (CPA), the other served as control.

administered in order to facilitate the growth of fairly immunosensitive tumors.

In only two experiments (IV, V) did remissions with long term survivors result from CPA treatment of non-immunized hosts. In the immunized subgroups CPA treatment effect was decidedly less effective with no long term survivors in experiment V. In the remaining experiments CPA caused only moderate remissions (IMST 5–22 days) for the non-immune groups. In these experiments the effect of CPA was improved in the immunized subgroups. The delicacy of the system is demonstrated in experiment V and VI in which similar tumor–host systems were used. In experiment V the effect of CPA was impaired in the immunized group; in experiment VI, employing a lower CPA dose and another tumor passage, CPA effectiveness was improved by preimmunizing the hosts.

Thus the therapeutic effect of CPA was augmented by pre-immunization in most cases. On the other hand, pronounced CPA effects, found in two experiments, seemed to be impaired by preimmunization.

*Effect of cyclophosphamide in neonatally Moloney virus infected mice.* In four experiments an attempt was made to exclude completely the host factor by growing the tumor in hosts neonatally infected with Moloney virus. Unfortunately, the effect of CPA was found to be only moderate in those tumor–host systems, it was possible to prevent development of cytotoxic antibodies by this pre-treatment. Thus discrimination between different subgroups was limited. In agreement with the experiments in animals with the immune response impaired by irradiation the therapeutic efficiency of CPA was found to be somewhat reduced (IMST reduced 0–8 days) in these possibly tolerant mice.

*Influence of tumor latent period on cyclophosphamide effect* (*Table* 4). — In order to vary tumor latent periods, different numbers of lymphoma cells were grafted. As in the preceding experiment CPA was administered on the first day when at least 50% of the animals carried palpable tumors. The size of the tumors at that time was similar in all animals. There was a considerably more pronounced CPA therapeutic effect on tumors treated 10–25 days after inoculation than on those treated after 5–6 days (i.e. those with a longer latent period were more sensitive).

*Effect of cyclophosphamide related to tumor immunosensitivity.* The immunosensitive lymphoma YAC and its immunoresistant subline YAC-IR were treated with CPA in five experiments (Table 5). Treatment of the immunosensitive line resulted in 75–100% long term survivors, but the effect on the resistant line was slight to moderate (IMST 11–35 days). This difference was greatly reduced in irradiated mice (experiment V).

*Table* 4. *Effect of cyclophosphamide on tumors with varying latent periods*

| Experiment No. | Tumor: Line | Tumor: Passage | Tumor: No. of cells grafted | Recipient mice: Strain | Recipient mice: Sex | CPA treatment day | Fraction of CPA treated mice with tumors at day of treatment | Median remission time (RT) (days) | Median survival time (days): Controls | Median survival time (days): CPA treated | Increase for CPA treated IMST (days) | Fraction of tumor free mice, alive, on day 100: Controls | Fraction of tumor free mice, alive, on day 100: CPA treated |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | YAC | 167 | $10^5$ | (A/Sn × C57B1)$F_1$ | ♀ | 10* | 4/4¶ | >88 | 20 | >100 | >80 | 0/4 | 3/4 |
| | | | $5 \times 10^7$ | (A/Sn × C57B1)$F_1$ | ♀ | 5 | 3/4‡ | 10 | 13 | 26 | +13 | 0/4 | 0/4 |
| II | YBA | 14 | $10^4$ | CBA | ♂ | 25† | 3/3§ | >70 | 65 | >100 | >35 | 0/3 | 2/3 |
| | | | $10^6$ | CBA | ♂ | 12 | 2/3¶ | >85 | 31 | >100 | >69 | 0/3 | 3/3 |
| | | | $4 \times 10^7$ | CBA | ♂ | 6 | 3/3‡ | 6 | 21 | 36 | +15 | 0/3 | 0/3 |

*CPA dose 170 mg/kg body weight.
†CPA dose 200 mg/kg body weight.
‡Median tumor diameter 2 mm.
¶Median tumor diameter 4 mm.
§Median tumor diameter 8 mm.

Different number of lymphoma cells were grafted to normal syngeneic recipients. On the first day when at least 50% of the mice carried palpable tumors each group was divided in two equal subgroups. One was treated with a single dose of cyclophosphamide (CPA), the other served as control.

*Table 5. Effect of cyclophosphamide on two lymphoma lines with different immunosensitivity*

| Experiment No. | Tumor: Line | Tumor: Passage | Tumor: No. of cells grafted | Recipient mice: Strain | Recipient mice: Sex | Recipient mice: Pretreatment | CPA treatment day No. | Fraction of CPA treated mice with tumors at day of treatment¶ | Median remission time (RT) (days) | Median survival time (days): Controls | Median survival time (days): CPA treated | Increase for CPA treated (IMST) (days) | Fraction of tumor free mice on day 100: Controls | Fraction of tumor free mice on day 100: CPA treated |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | YAC | 167 | $10^5$ | (A/Sn × C57B1)$F_1$♀ | | | 10† | 4/4 | >88 | 18 | >100 | >82 | 0/4 | 3/4 |
| | YAC-IR | 24 | $10^6$ | (A/Sn × C57B1)$F_1$♀ | | | 7 | 4/4 | 10 | 10 | 24 | +14 | 0/4 | 0/3 |
| II | YAC | 176 | $10^6$ | (A/Sn × C57B1)$F_1$♀ | | | 11† | 4/4 | >82 | 29 | >100 | >71 | 0/4 | 3/4 |
| | YAC-IR | 20 | $10^4$ | (A/Sn × C57B1)$F_1$♀ | | | 10 | 2/4 | 12 | 17 | 31 | +14 | 0/4 | 1/4 |
| III | YAC | 170 | $10^5$ | (A/Sn × C57L)$F_1$♀ | | | 17‡ | 1/2 | >75 | 45 | >100 | >55 | 0/2 | 2/2 |
| | YAC-IR | 25 | $10^5$ | (A/Sn × C57L)$F_1$♀ | | | 11 | 2/2 | 50 | 17 | 28 | +11 | 0/2 | 1/2 |
| IV | YAC | 176 | $10^6$ | (A/Sn × C57L)$F_1$♀ | | | 11‡ | 4/4§ | >86 | 37 | >100 | >63 | 0/4 | 4/4 |
| | YAC-IR | 23 | $10^6$ | (A/Sn × C57L)$F_1$♀ | | | 8 | 3/4 | 41 | 15 | 50 | +35 | 0/4 | 0/4 |
| V | YAC | 163 | $10^6$ | (A/Sn × C57B1)$F_1$♂ | | IRR | 7* | 2/3 | 17 | 13 | 37 | +34 | 0/4 | 0/3 |
| | YAC-IR | 24 | $10^6$ | (A/Sn × C57B1)$F_1$♀ | | IRR | 11 | 4/4 | 12 | 10 | 20 | +10 | 0/4 | 0/4 |

*CPA dose 150 mg/kg body weight.
†CPA dose 170 mg/kg body weight.
‡CPA dose 200 mg/kg body weight.
¶The median tumor diameter ranged 2–8 mm. Differences of 2 mm or less were noted between YAC and YAC-IR groups except in the § indicated experiment.
§The median tumor diameter of the YAC group was 3 mm greater than for the YAC-IR group.

The immunosensitive lymphoma line YAC and its immunoresistant subline YAC-IR were grafted to syngeneic recipients. In one experiment all recipients were irradiated (400 r) (IRR). On the first day, when at least 50% of the mice carried palpable tumors, each group was divided in two equal subgroups. One was treated with a single dose of cyclophosphamide (CPA), the other served as control.

Suggestive for a correlation between CPA effect and tumor immunosensitivity are the results on other tumors also. The immunosensitivity of the tumors in Table 3 is reflected in the prolongation of MST by preimmunization. Thus, YAC, YALB, YBA and YDAG are probably the most immunosensitive. Compared with the others all these tumors, except YDAG, seemed to be more affected by CPA, in mice in which no manipulation with the immune response was introduced (Tables 2 and 3).

*The effect of cyclophosphamide on the development of cytotoxic antibodies.* This was followed in experiment VI (Table 2, Fig. 1). In CPA treated mice there was a delay in the appearance of antibodies and titers were lower. Low antibody levels were found even at long periods after immunization. At day 51 in experiment II (Table 5) the mean cytotoxic index for four animals was 0·67 in the CPA treated while in the controls it was 0·84; a difference significant at the 5% level.

In some experiments an *effect of cyclophosphamide on generalization of tumor growth* was observed (Table 6). Lymphomas which were originally localized more often widely disseminated on recurrence after CPA treatment. As change in inherent tumor-cell invasiveness seems unlikely to have occurred during the short periods of remission (6–17 days), it is probable that disseminations were a sign of CPA induced breakdown of host factors which could control tumor spread.

## DISCUSSION

Both treatment with cytostatic drugs and the host reactivity against an antigenic tumor can destroy tumor cells. The immune response might augment the drug effect unless its potency to destroy tumor cells is insignificant in relation to the antitumor effect of the drug. Such a relative inadequacy of the host response might either by inherent or due to the immunodepressive activity of the drug. The influence

*Table 6. Effect of cyclophosphamide on generalization of tumor growth*

| Experiment, Table No. | Tumor | Pretreatment of hosts | Fraction of animals with generalized lymphoma growth/all animals with tumors | |
|---|---|---|---|---|
| | | | No. CPA treatment | Recurrences in CPA treated |
| III, 2 | YAC | — | 1/4 | 2/2 |
| | | IRR | 1/4 | 2/3 |
| V, 2 | YAC-IR | — | 0/4 | 1/4 |
| | | IRR | 4/4 | 4/4 |
| III, 3 | YALB | IRR | 0/4 | 2/3 |
| | | IM+IRR | 0/3 | 1/1 |
| VIII, 3 | YBC | IRR | 1/4 | 5/5 |
| | | IM+IRR | 0/3 | 0/2 |
| X, 2 | YDYA | — | 1/3 | 3/3 |
| | | IRR | 2/4 | 4/4 |

Lymphomas were grafted to normal, whole body irradiated (400 r) (IRR) or pre-immunized (IM) syngeneic recipients. The experiments are recorded in the tables noted. This table lists the experiments in which there was a difference in the fraction of animals with generalized lymphoma growth in the CPA treated group as compared to the controls. Generalization is defined as equilateral enlargement of inguinal and axillary lymph nodes and/or spleen without any tumor at the site of inoculation.

of immune factors on the therapeutic outcome of CPA treatment were investigated in this series of experiments.

Reliable effective cytostatic activity was critical for the disclosure of any significant host influences. A noticeable improvement of therapeutic results in normal immunoreactive mice compared to irradiated mice was mainly demonstrated in experiments with a cytostatic effect resulting in an IMST of at least 12 days in the immunological inert group. A similar tendency was evident in the experiments with pre-immunized mice. This suggests that the drug has to decrease the tumor cell population to a certain minimum level, characteristic for each tumor–host system, to permit any substantial influence of immune factors on the therapeutic outcome.

The effect of CPA was weaker in irradiated mice. Since not only IMST but also RT were longer in the non-irradiated mice the decreased effect can not be ascribed to a higher toxicity of CPA in the irradiated group. Specific immunodepression was attempted by employment of neonatally MLV inoculated mice, considered to be tolerant. In these experiments too treatment of the immunologically non-reactive hosts was less efficient. Due to a low CPA effect on the non-tolerant animals, however, the system was not very discriminative.

In one experiment (VII, Table 2) in this series, the immunodepressive action of the drug was verified (Fig. 1). It is evident that in spite of the depression of the humoral antibody response by CPA, the grade of immunoreactivity still present permitted the therapeutic effect to be far superior to that in the immunological inert group. Except in the experiments in which CPA treatment was followed by disseminated growth after a variable regression following CPA treatment, the immunodepressive activity of the drug did not decisively dominate the therapeutic outcome.

The immunoreactivity of mice exposed to growing tumor cells, seemed to contribute to the markedly better effect of CPA on the immunosensitive lymphoma line YAC than on YAC-IR, a subline selected for immunoresistance. Unless the sensitivity of the cells to CPA was altered in parallel with the change in immunosensitivity, the different sensitivity to the host response would seem the likely explanation of these findings. In support of this interpretation the disparity in CPA effect was greatly reduced in irradiated mice.

Augmented host sensitization was attained either by inoculation of irradiated tumor cells prior to transplantation of the tumor or by grafting a small number of tumor cells. In the latter case sensitization will develop concomitant with a prolonged period of tumor growth.

CPA treatment was most effective in animals with long tumor latent periods. In fact the augmenting influence of immunoreactivity on the therapeutic effectiveness of CPA was best demonstrated in this system. While animals treated on day 5 or 6 had IMST of 13–15 days, the same treatment at 10–25 days after tumor inoculation resulted in 75–100% long term survivors.

The influence of sensitization with irradiated tumor cells was more complex. In the majority of experiments sensitized animals responded better to CPA than unsensitized. Pronounced CPA effects, however, found in two experiments, seemed to be impaired by sensitization. The mechanism of this phenomenon is unclear at the moment.

The number of tumor cells present in a palpable tumor exceeds the eradicating capacity of a single dose of CPA. The occurrence of long term survivors may indicate that the small fraction of remaining tumor cells may be inhibited by host factors. It is clear from the experiments that increased immunoreactivity results in improved therapeutic effect although a few unpredictable exceptions to this rule did occur. For demonstration of substantial immunological influence pronounced drug effect reducing the number of tumor cells to a level within the capacity of the immune factors seems to be a pre-requisite. If the cytostatic effect of the drug is inefficient, its immunodepressive action might prevail with the consequence of disseminated growth after an insignificant remission (experiment X, Table 2; VIII, Table 3).

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## SUMMARY

*The influence of immune factors on the therapeutic effect of cyclophosphamide (CPA) on 13 different Moloney viruses (MLV) induced lymphomas grafted to syngeneic mice with different immunological reactivity has been investigated. The animals were treated with a single dose of CPA on the first day when 50% of the animals developed palpable tumors. The immunological reactivity of the host was decreased by irradiation or by neonatal MLV inoculation and stimulated by previous inoculation of irradiated lymphoma cells or low tumor cell inocula which resulted in long latent periods prior to tumor development. In accordance with the threshold character of the immune response noticeable differences in treatment effect between groups of different immunoreactivity was only apparent when the treatment effect, in the least immunoreactive group, was pronounced. An increased immunoreactivity resulted in an improved therapeutic effect with the exception of two experiments in which the animals were sensitized by irradiated tumor cells. A highly immunosensitive lymphoma cell line was markedly more affected by CPA than an immunoresistant variant derived from it. The general picture emerging from these results suggests that immunoreactivity against an MLV lymphoma may, under certain circumstances, augment the therapeutic efficiency of CPA.*

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# Virus Particles in a Transplantable Rat Mammary Tumor of Spontaneous Origin*

H. C. CHOPRA, A. E. BOGDEN, I. ZELLJADT and E. M. JENSEN

*The John L. Smith Memorial for Cancer Research, Chas. Pfizer & Company, Inc., Maywood, New Jersey 07607 and Mason Research Institute, Worcester, Massachusetts 01608, U.S.A.*

## INTRODUCTION

DURING serial transplantation of a subline of the spontaneous Huggins mammary fibroadenoma A [1], there was a gradual microscopic change in the morphology of the tumor to that of an adenocarcinoma. This subline, designated R-35, is morphologically distinct and has been stabilized and readily transplantable in Sprague–Dawley: (SD) stock female rats since 1959.

Electron microscopic study was undertaken in search for virus particles which may be etiologically related to the development of the mammary adenocarcinoma. Investigations were also undertaken to propagate virus particles in tissue culture as well as to isolate this virus by density gradient fractionation. The virus particles detected in the R-35 mammary adenocarcinoma are morphologically of C-type and are now being readily propagated in tissue culture for biological and immunological studies.

## MATERIAL AND METHODS

The R-35 mammary adenocarcinoma has been routinely transplanted in 45–60-day old female rats of non-inbred Sprague–Dawley: (SD) stock. Tumor grafts, 1 or 2 Stadie slices $10 \times 10 \times 1$ mm, are implanted subcutaneously in the supra-scapular and/or supra-sacral region. Palpable tumor-growth is evident in 5–6 days with progressive lethal growth occurring in 85% of implanted animals. Mean survival time has been 23·4 days with a standard deviation of $\pm 4 \cdot 6$ days.

*Electron microscopy*

Small pieces of R-35 tumor tissue were fixed in glutaraldehyde and were postfixed in chrome-osmium. Similarly, cell cultures of the tumor were detached from the glass and fixed in glutaraldehyde and chrome-osmium. The samples were dehydrated in successive changes of 50% ethyl alcohol (containing 2% uranyl acetate) and 70, 95 and 100% ethyl alcohol. The dehydrated samples were placed in various proportions of propylene oxide and the embedding medium. The material was embedded in Araldite (Fluka) or Epon and allowed to harden in an oven at temperatures of 37°C and 60°C. Thin sections were cut with a diamond knife on an LKB ultramicrotome. The sections were mounted on carbonized formvar membranes on 200 mesh copper grids. The sections were stained with lead citrate [2] and examined in a Siemens Elmiskop Model I-A electron microscope.

*Tissue culture*

Primary cultures of rat mammary tumor (R-35) were established by mincing the tissue and trypsinizing the mince three times. Cells were pooled, washed and resuspended in RPMI

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medium 1640, supplemented with 20% fetal calf serum, in 75 $cm^2$ plastic-tissue culture flasks. Each flask was seeded with $2 \times 10^6$ viable cells per ml. Cultures were incubated at 36°C and passaged at regular intervals of 3–4 days in 1640 medium. The cell cultures were developed as monolayers. For electron microscopy, the cells were trypsinized, sedimented by gentle centrifugation and processed as described above. The supernatant fluids from these cultures were concentrated by centrifugation at 30,000 **g** for one hr. The concentrated virus suspension was diluted 1:2 with 2% phosphotungstic acid at pH 5·0 [3]. The specimens were prepared for negative staining-electron microscopic study by applying carbon-coated grids (200 mesh) to the surface of the virus-phosphotungstic acid suspension and allowing to dry at room temperature.

## RESULTS

The R-35 tumor is a lobulated, nodular well vascularized subcutaneous growth covered with a loose, thin connective tissue capsule (Fig. 1). Microscopically, it appears as a very rapidly growing epithelial structure containing many small acini (Figs. 2–4). These usually contain no secretion but occasionally one finds an acidophilic homogeneous material. In more advanced stages of tumor growth the tumor cells are usually arranged in multiple layers of cuboidal epithelial cells, with the surface layers in various stages of necrosis and sloughing. The lumen of the acini are often irregular and filled with cells in all stages of disintegration mixed in with large numbers of histiocytes. The stroma may vary from 10 to 50% while the extension of necrosis depends on the portion of tumor examined and the age of the growth.

As revealed by electron microscopy, the R-35 mammary adenocarcinoma is characterized by glandular cell types (Fig. 5). These glandular cells form the innermost layer of the epithelium, the free margins of which border on duct-like spaces. The peripheral layer of cells in the nests of tumor is usually surrounded by myoepithelial cells which is bounded by a basement membrane. The glandular cells are slightly flattened and are joined to one another by junctional complexes. Nuclei are large in proportion to the amount of cytoplasm. Older tumors showed necrotic nests of tumor cells (Fig. 6). Secretive tumor cells are commonly observed to border lactating ducts.

Thin sections of six successive tumor transplants were examined with the electron microscope. These tumors have invariably revealed the presence of virus particles which were observed in various stages of replication (Figs. 7–10). The virus particles were found in varying numbers at different stages of tumor growth; early stages as well as older necrotic tumor cells revealed a few particles. The particles replicate by budding from the cell membrane and were localized in the extracellular spaces or in the cytoplasmic vacuoles. The extracellular forms were morphologically of C-type [4] having a diameter of 90–100 mμ, with an outer unit membrane and a densely stained central nucleoid (Fig. 10). The virus particles develop by a budding process from the cell surface as a crescent of high electron density (Fig. 7) and are released extracellularly (Figs. 8 and 9).

In tissue culture, the tumor cells showed a characteristic morphology (Figs. 11 and 12) and occasionally the cells were observed in a mitotic phase. The nuclei were rounded or crescentic but with marked surface irregularities and indentations. The nucleoplasm contained prominent nucleoli and showed marginal distribution of dense chromatin. The cytoplasm was relatively abundant in free ribosomes. Scanty elements of rough-surfaced endoplasmic reticulum, mitochondria and poorly developed Golgi components were present. The cells showed deposits of secretory material in the cytoplasm which was heavily stained with lead citrate. Occasionally, cells with intracytoplasmic ductules, which were lined with microvilli, were observed. No significant amount of secretion was noticed in these cells.

The tissue culture cells were observed to be replicating C-type particles at a much greater frequency than the original tumor cells. The virus particles were found extracellularly in the depleted tissue culture medium (Fig. 14). These particles, when examined by phosphotungstic acid-negative staining technique, exhibited the morphology of C-type virus consisting of a head and tail (Fig. 15).

Sucrose-density gradient purification of the particles from the tumor as well as from tissue culture medium yielded a larger number of virus particles at buoyant density of 1·14–1·18 g/$cm^3$. Preliminary biological experiments to inoculate concentrated virus particles in newborn Sprague–Dawley rats and BALB/c mice have not yielded any results during a one year period. This may be attributed to the low quantity of virus particles inoculated in these animals. However, tissue culture cells inoculated intraperitoneally into newborn rats pro-

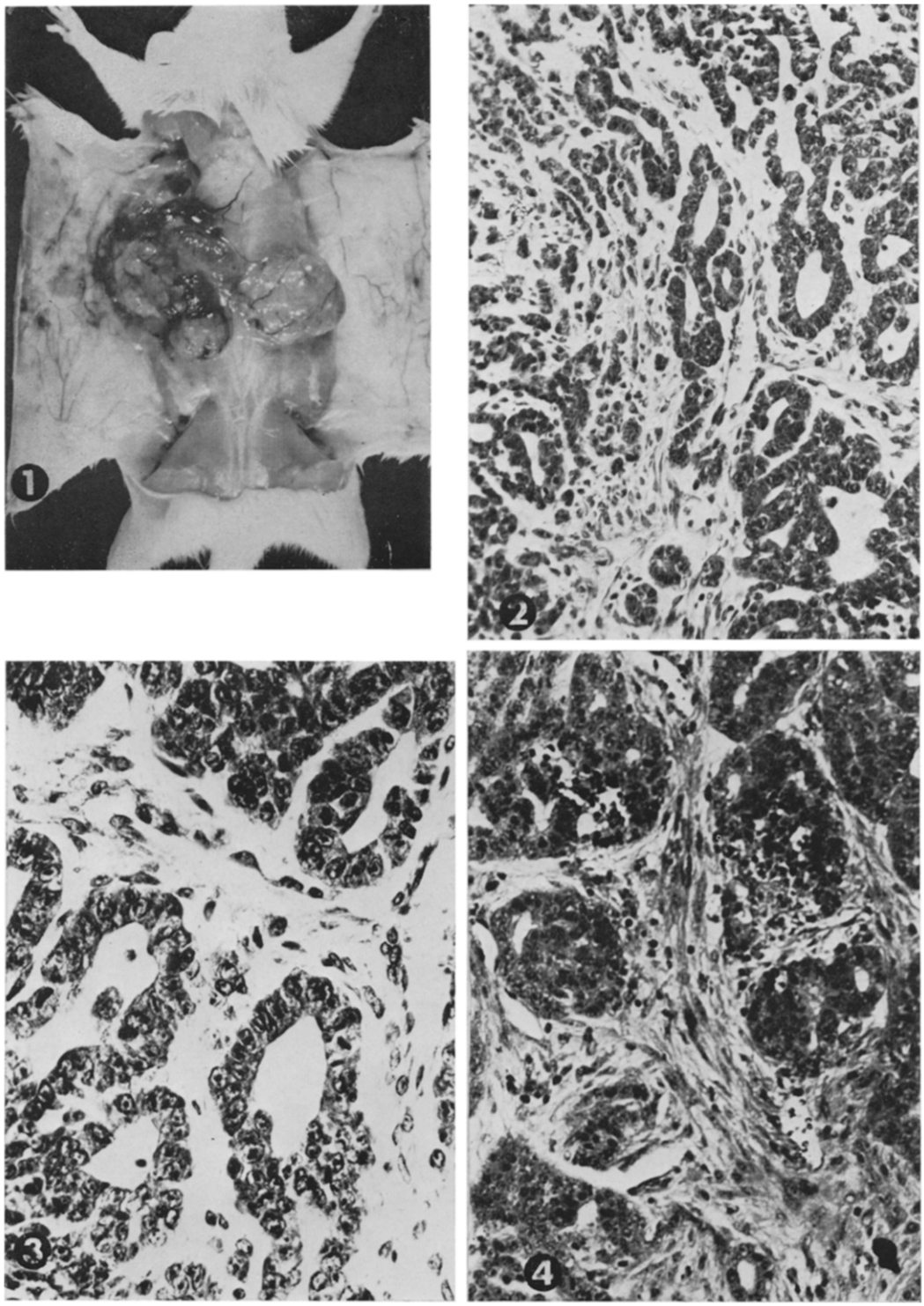

*Fig.* 1. *Subcutaneous implants of the R-35 tumor infiltrate the muscle at the site of inoculation to very moderate extent. They are firm, somewhat lobulated, covered with a thin connective tissue capsule and not generally adhered to the skin except larger, older implants which have become ulcerated. On cut surface, the tumor is yellow-white and contains many small cavities filled with pus-like material. Metastases from the implant seldom occur.*

*Fig.* 2. *The tumor consists of irregularly shaped acini generally lined with a single layer of epithelial cells. Many larger acini contain necrobiotic cells presumably desquamated from the epithelial lining; milk is never seen within acini. A loose connective tissue stroma usually comprises about one-fifth of the tumor and shows considerable vascularization. Hematoxylin and eosin* (×75).

*Fig.* 3. *A portion of the tumor in Fig.* 4 *shows the cuboidal epithelial cells lining the acini, the round, distinct nucleus has marginated chromatin and a prominent single nucleolus; the foamy violaceous cytoplasm. Desquamated epithelial cells can be seen at lower right. Several mitotic figures indicate a relatively rapid rate of cellular division. Hematoxylin and eosin* (×150).

*Fig.* 4. *More recent transplants of the R-35 tumor have shown a greater tendency for epithelial cells to pile up filling the acini. Such acini often contain large areas of necrobiotic cells and some neutrophils, and much of the tumor is composed of this necrotic material. Hematoxylin and eosin* (×75).

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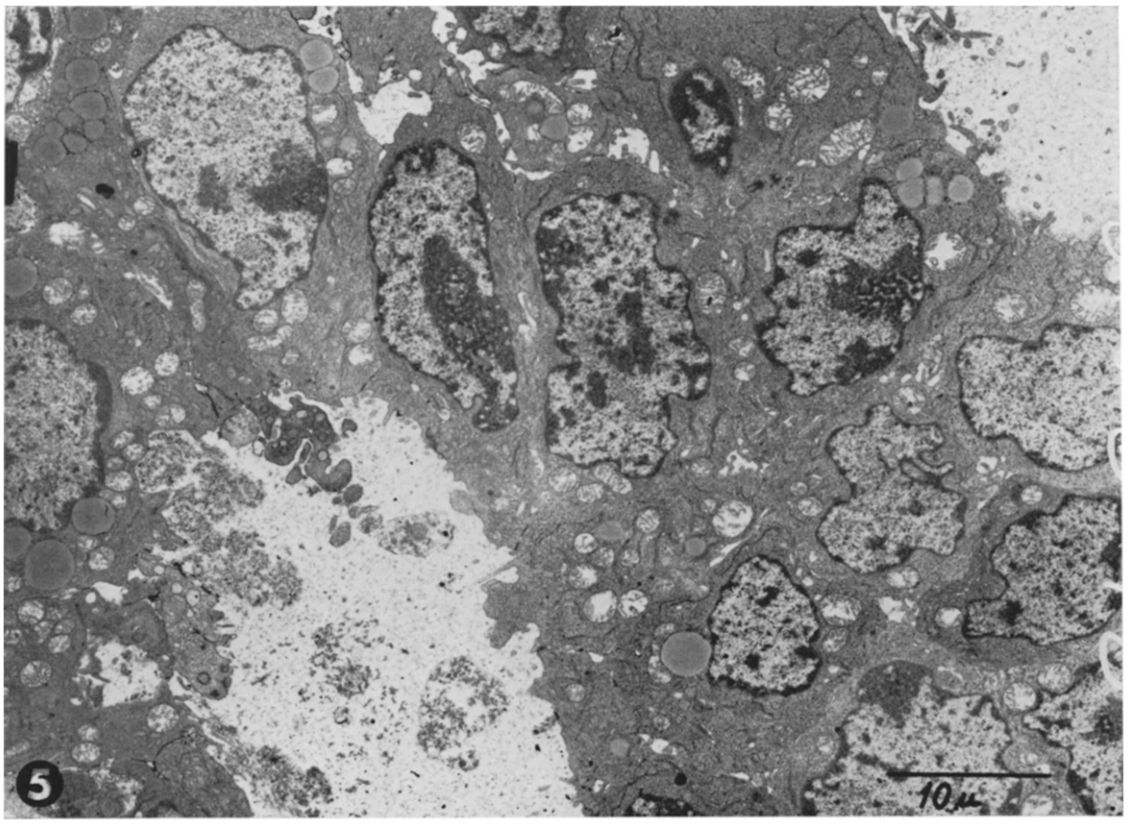


*Fig.* 5. *Electron micrograph showing general morphology of R-35 rat mammary tumor. The tumor cells border the secretory ducts* ( × 2000).

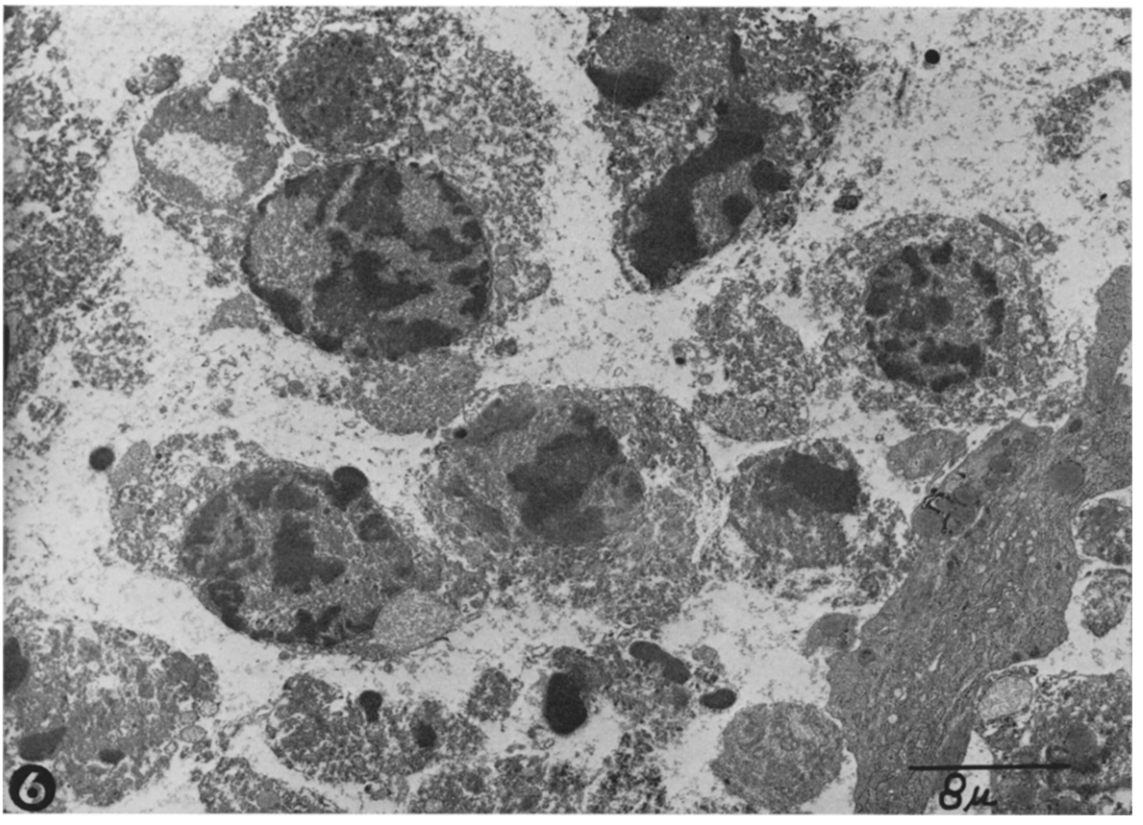


*Fig.* 6. *The tumor cells from late stage of R-35 carcinoma showing necrosis* ( × 2670).

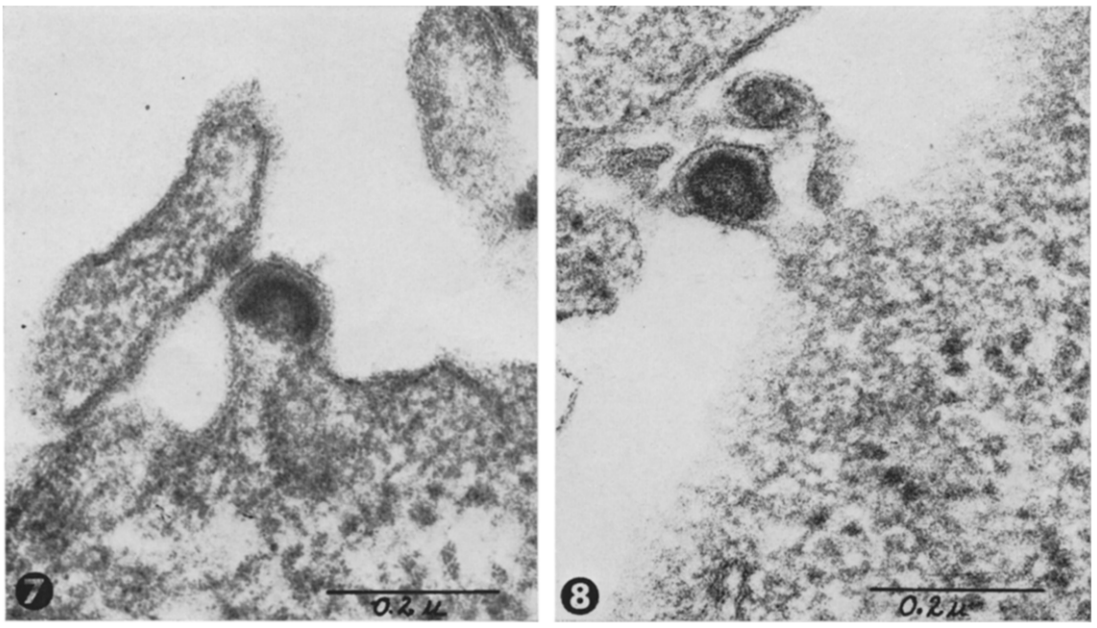


*Fig.* 7. *Early budding C-type particle from the cell surface* ($\times$ 142,000).
*Fig.* 8. *Later budding C-type particle in which the intermediate membrane between the nucleoid and the outer membrane of a particle is almost complete* ($\times$ 142,000).

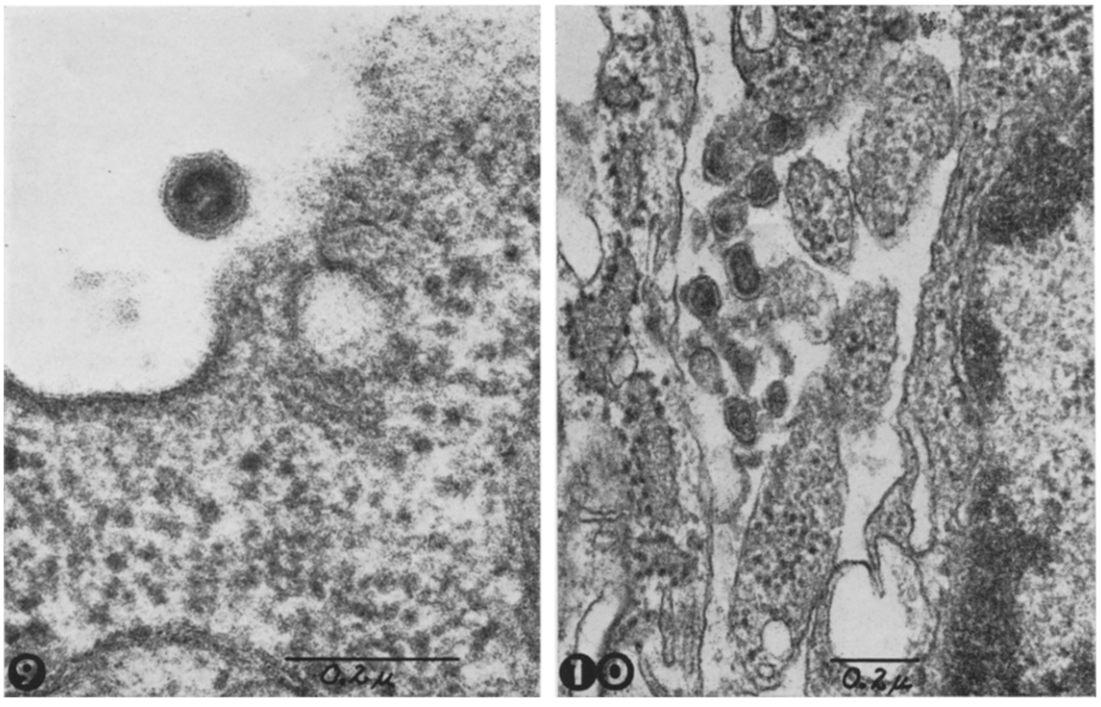


*Fig.* 9. *Immature C-type particle having an outer membrane and an intermediate membrane, with a partially dense nucleoid* ($\times$ 142,000).
*Fig.* 10. *A group of mature C-type particles in the intercellular space* ($\times$ 71,000).

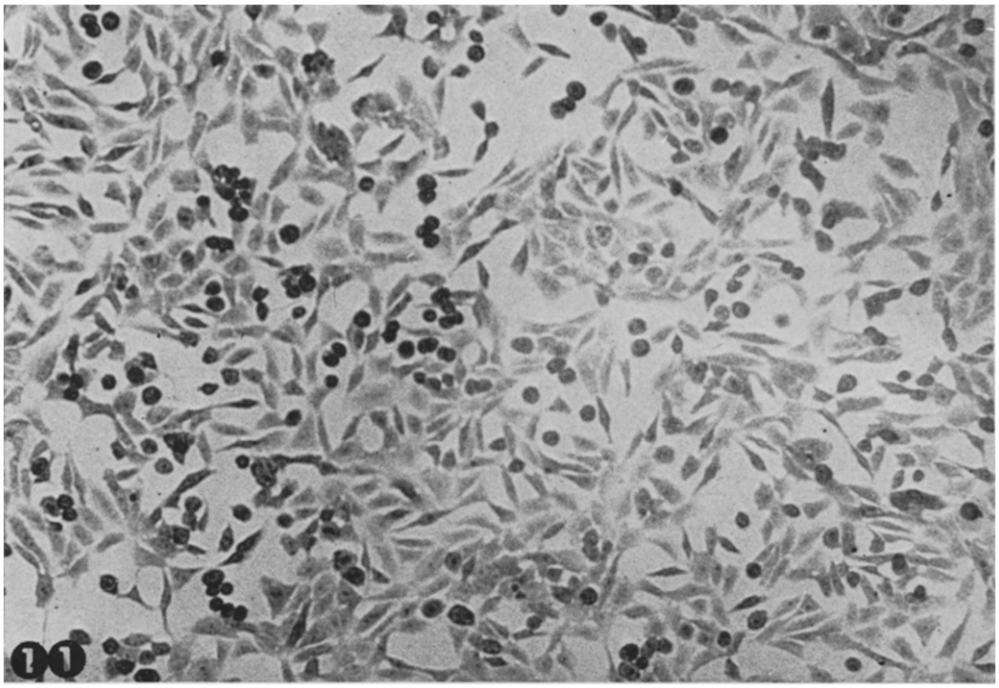

*Fig.* 11. *A light micrograph of tumor cells growing as monolayers in a tissue-culture bottle. A few rounded cells in various stages of mitosis are also observed.*

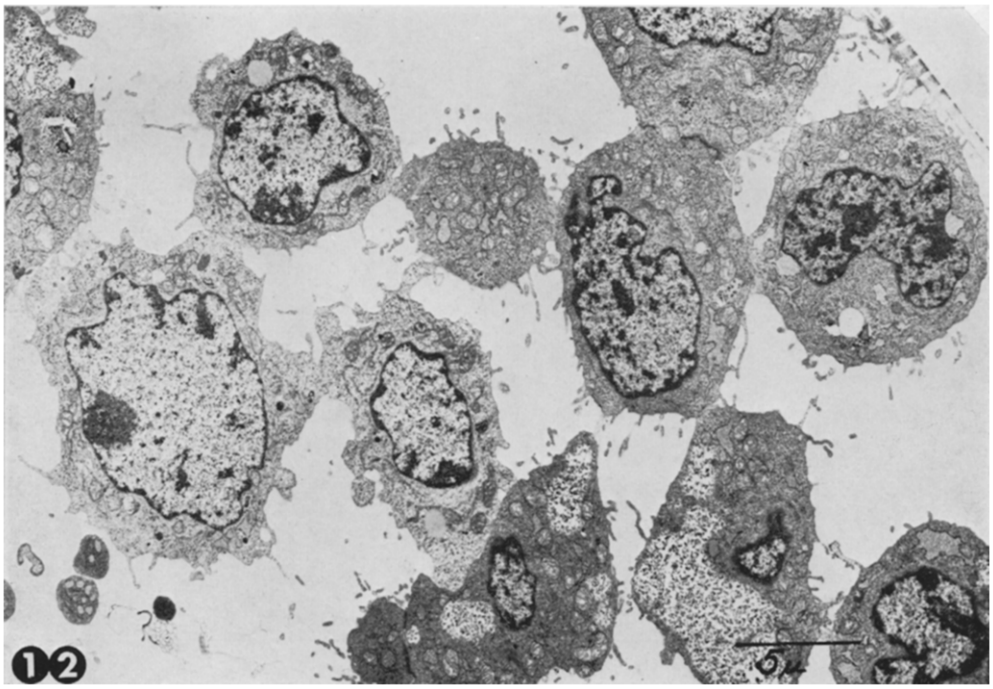

*Fig.* 12. *Electron micrograph of R-35 tumor cells in tissue culture. The cells exhibit intracytoplasmic secretory material* ( $\times$ 4000).

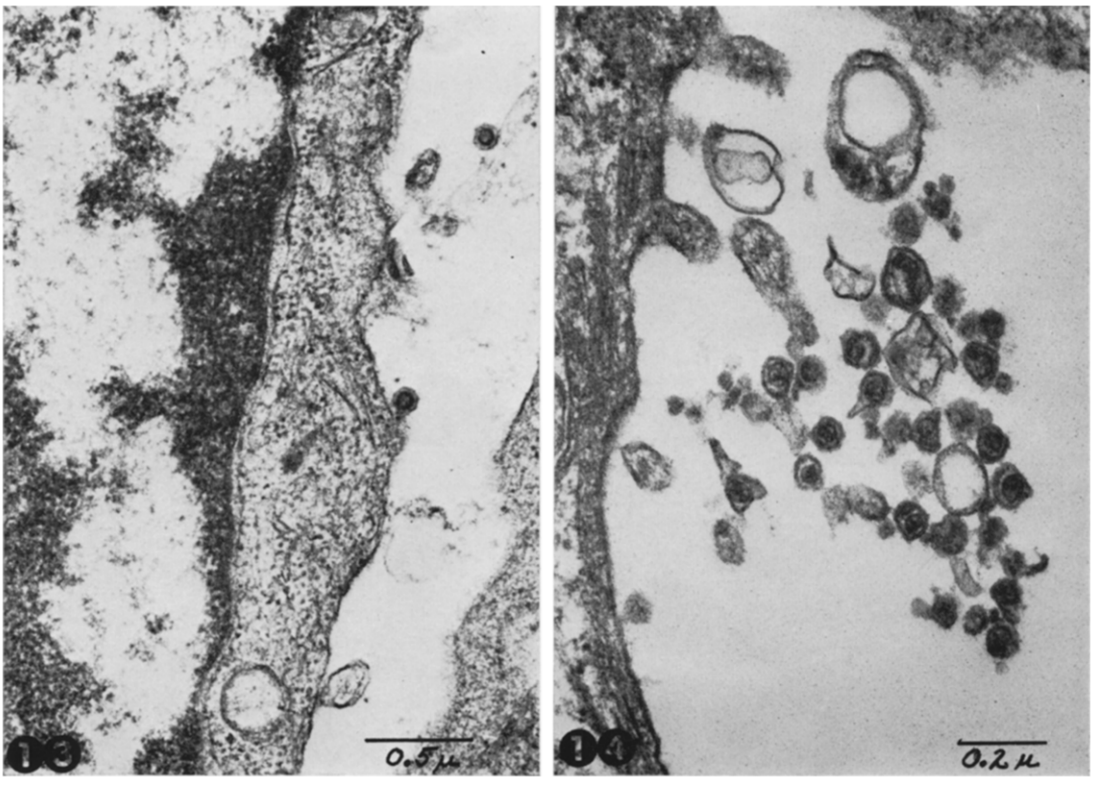


*Fig.* 13. *Electron micrograph showing C-type virus particles in a tumor induced by inoculation of tissue-culture cells (R-35) in a newborn Sprague–Dawley rat* (×35,400).
*Fig.* 14. *Extracellular C-type particles replicating in R-35 tumor tissue culture cells. A few "small particles" can also be detected* (×71,000).

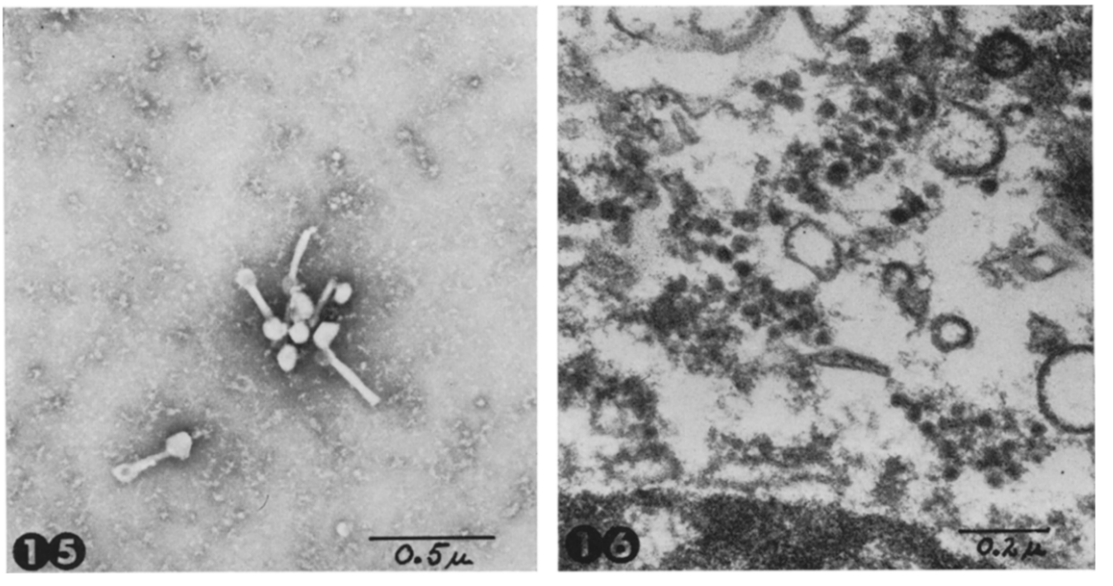


*Fig.* 15. *Electron micrograph of negatively stained (PTA) virus particles recovered from R-35 mammary tumor cell culture. The particles show typical head and tail morphology of C-type virus* (×41,400).
*Fig.* 16. *Small virus-like particles observed in rat-mammary tumor cells (R-35). The particles consisting of a unit membrane and a dense core measure* 20–50 mμ *in size* (×71,000).

duced anaplastic adenocarcinoma which contained C-type particles (Fig. 13).

Another structure observed to be associated with these rat mammary tumors is a distinct, small particle which measures 20–50 mμ in size (Fig. 16). The particle consists of an outer limiting membrane and an electron dense nucleoid. The small particles were observed individually or in clusters in the cytoplasm of cells, both in original tumor and in tissue culture cells.

## DISCUSSION

The induction of rat mammary tumors by chemical carcinogens has been reported by various investigators [5–8]. No virus particles were reported in these tumors. Recently, however, Engle *et al.* [9] described C-type particles in the transplantable carcinoma induced in North-West Sprague–Dawley rats by X-irradiation. Attempts have also been made to adapt mouse mammary tumor virus in rats to produce mammary tumors but, so far no conclusive results have been achieved [10].

In the present investigations, a transplantable subline of the spontaneous Huggins mammary fibroadenoma A, which transformed into a mammary adenocarcinoma during prolonged serial transplantation, was found to contain virus-like particles. Morphologically, these particles are similar to those observed by Engle *et al.* [9]. Similar electron microscopic study of a wide variety of transplantable rat mammary tumors, induced by chemical carcinogens, has revealed the presence of C-type particles [11]. Since these particles resemble C-type virus which cause murine [12], avian [13] and feline [14] leukemias, the oncogenic nature of this virus is probable. However, their etiological role in rat mammary tumors remains to be established.

There is the possibility that this virus may be a contaminant mouse-leukemia virus, but the virus particles observed in rat mammary tumors may be etiologically related to the tumors. Negative CF tests with broad reacting antiserum against murine-leukemia viruses suggest that the virus associated with rat mammary tumors (R-35) is antigenically unrelated to known murine-leukemia viruses [15]. Significantly, also, unlike the murine leukemias, no C-type virus particles could be detected in other organs of the rats bearing mammary tumors. As mentioned, it is of great interest that various chemically induced rat mammary tumors also harbour C-type virus particles.

The etiology of mouse mammary cancer has been established to be associated with virus particles of B-type while murine-leukemia-sarcoma complex is caused by C-type particles. This morphological classification of virus particles in the mouse system has been suspected in other animal species. Both types of particles have common characteristics in the process of budding from the cell surface and consisting of an outer unit membrane enclosing a nucleoid. However, the slight difference in size, location of the nucleoid and surface spikes on B particles would probably be not significant enough to determine the specificity of the malignancy caused by these oncogenic viruses. It is quite likely that particles of C-type observed in rat mammary tumors may be the causative agent.

The small particles observed in R-35 mammary tumors measure 20–50 mμ. The particles occur singly or in clusters. Identical structures have been reported by Feller and Chopra [16] in human breast cancer, by Moses *et al.* [17] to be associated with infectious mononucleosis and by Olson *et al.* [18] in Bowen's disease. Although the exact nature of these particles awaits further definition, their ultramorphology consisting of a unit membrane and a dense core is suggestive of their viral nature. However, their relationship to rat mammary cancer has not been demonstrated.

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## SUMMARY

*The rat mammary adenocarcinoma (R-35), which developed as a subline of the Huggins spontaneous fibroadenoma A during serial transplantation, is readily transplantable in female rats of Sprague–Dawley: (SD) stock. Electron microscopic studies of this tumor have revealed the presence of C-type virus particles, both* in vivo *and* in vitro, *which were not detected in other organs of rats bearing mammary tumors. The etiological significance of these virus particles is still undetermined.*

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# Tumor Growth Inhibiting Effect of JB-1 Ascitic Fluid—I

## An *In Vivo* Investigation*

P. BICHEL

*Cancer Research Institute, The Danish Cancer Society*
*Radiumstationen, DK8000 Aarhus C, Denmark*

### INTRODUCTION

IT IS a well known fact that several transplantable tumors show a decreasing growth rate as the tumor increases in size. This includes solid tumors [1, 2] as well as ascites tumors [3–5].

In 1957 Goodman [6] found that the total mass of tumor tissue was the same at death no matter whether the mice were suffering from one or more tumors. Following strangulation or excision of one tumor in a mouse transplanted with two identical tumors at the same time, it was observed that the other tumor accelerated the growth. At death the total mass of tumor tissue was the same as in the controls.

Recently Burns [7] showed that aspiration of most of the ascites tumor from mice bearing a 14-day old Ehrlich ascites carcinoma was followed by a second increase of tumor growth in the mice, and Burns [8] suggests the following: "Evidence exists which indicates the possibility that some tumors may regulate their own growth as normal or non-neoplastic tissues do by the production of a homologous specific mitotic inhibitor". This report presents data which may be interpreted as support for the hypothesis of self-inhibition of tumor growth.

### MATERIAL AND METHODS

In these investigations a transplantable ascites tumor JB-1 [9] has been used. The tumor is a hypotetraploid plasmocytoma which in 1950 appeared spontaneously in the AK/A strain of mice (inbred in this laboratory since 1943). The plasmocytoma grows both as a solid and as an ascites tumor and has been maintained through syngeneic passages. After intraperitoneal inoculation of $5 \times 10^6$ cells the mice develop a heavy ascites (10–15 ml) and will die about 20 days later.

The total number of tumor cells reaches a maximum of $10^9$ about 14–16 days after the inoculation.

AK/A mice about two months old were used as hosts and the animals were kept on Althromin(R) and tap water *ad libitum*.

Aspiration of the ascites tumor was performed by percutaneous puncture with a fine needle. After the penetration of the skin a subcutaneous route of about 1 cm was followed before perforating the peritoneum in order to avoid leakage.

The mitotic index (M.I.) (percentage of tumor cells in mitosis) was determined by counting 1000 tumor cells on smears.

The colchicine method was used to demonstrate differences in growth rates. Each mouse received intraperitoneally 1 mg/kg of N-deacetyl-N-methylcolchicine (Colcemid(R)) in aqueous solution, and the percentage of ascitic tumor cells in mitosis was determined every hour for the following 5–6 hr. After that time the occurrence of micronuclei indicated Colcemid intoxication.

The cell free tumor ascites was obtained by aspiration from mice bearing 10 day-old ascites

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tumors with subsequent centrifugation of the ascites tumor 3000 rpm for 10 min. Non-tumorous mice were anesthesized with Nembutal Natrium(R) 70 mg/kg, and a sterile nylon sponge was placed intraperitoneally through a small longitudinal incision in the abdominal wall. The incision was sutured with Mersilene. The mice were killed by cervical dislocation 3–4 days later, and the exudate from the sponge was centrifugated for 10 min at 3000 rpm. Using this method it was possible to obtain 1·5–2·5 ml of cell free "normal ascites" from one mouse.

## RESULTS

The mitotic index (MI) of the ascitic tumor was followed from the inoculation of the tumor until the terminal stage of the animal (Fig. 1).

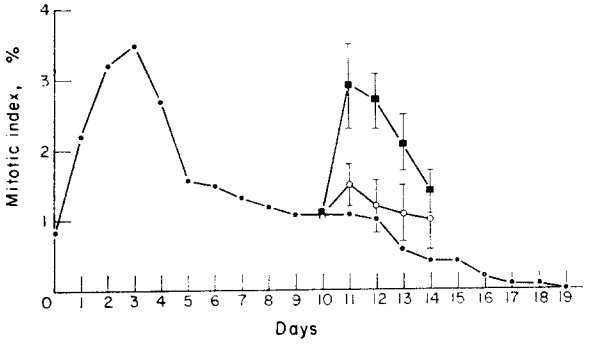


*Fig. 1. Mitotic counts in ascites tumors after inoculation with* $5–7 \times 10^6$ *JB-1 cells* ●—● *(mean of 5 different determinations). Percentage of mitotic cells following aspiration* ■—■ *(mean of* $20 \pm SE$ *different determinations). Aspiration and subsequent injection of tumor ascitic fluid is indicated by* O—O *(mean of* $13 \pm SE$ *different determinations).*

Three days after the inoculation of the tumor a maximum of 3–4% is reached followed by a steep fall to 1·5% on day 5 or 6 when the curve flattens out. The decrease of the M.I. will continue until the death of the animal.

Aspiration of the ascites tumor, as much as possible, was performed 10 days after the inoculation of the tumor, and the following days a considerable increase of the M.I. was seen (Fig. 1). This observation is principally in accordance with the rise in the incorporation of tritiated thymidine into DNA seen after aspiration of most of the tumor from mice bearing 14-day old Ehrlich ascites carcinomas [7].

Although several mechanisms are possible, it is tempting to explain the growth retardation of the fully developed tumor and the recurrent proliferation of the tumor seen after aspiration by a self-inhibition hypothesis.

Following this theory the individual ascites-tumor cells must receive some information from the neighboring tumor cells concerning the actual state of development of the ascites tumor. This information must be humoral and mediated via the ascitic fluid because of the suspended state of the ascites tumor cells.

As much as possible of the JB-1 ascitic tumor was removed from mice bearing 10 day-old tumors, and twice daily (9 a.m. and 3 p.m.) the animals were injected intraperitoneally with 1 ml of freshly prepared cell free tumor ascitic fluid. It will appear from Fig. 1 that there was a great difference between the M.I. of the animals subjected to aspiration and those receiving the tumor-ascitic fluid. No difference was seen between the M.I. of the tumor cells from the "aspirated" mice and the mice that twice daily received 1 ml of the "normal ascites" previously described.

Treatment with Colcemid was performed on the second day after the aspiration. It was obvious that much more of the tumor cells in metaphases were accumulated in the mice subjected to total aspiration and in those receiving the "normal ascites" after the aspiration than in the mice which received the tumor ascites after the aspiration and in the controls (Fig. 2).

It will be seen from Figs. 1 and 2 that the

*Table 1. Mice subjected to aspiration of the ascitic tumor 10 days after the inoculation (group I). Mice subjected to aspiration of the ascitic tumor 10 days after the inoculation followed twice daily by injection of 1 ml of cell free ascitic fluid (group II)*

| | | Mean value of M.I.±S.D. | | | |
|---|---|---|---|---|---|
| | No. of animals | 1 day after aspiration | 2 days after aspiration | 3 days after aspiration | 4 days after aspiration |
| Group I | 20 | 2·9±0·6 | 2·7±0·4 | 2·1±0·4 | 1·4±0·3 |
| Group II | 13 | 1·5±0·3 | 1·2±0·4 | 1·1±0·4 | 1·0±0·4 |

M.I. = Mitotic index.
S.D. = Standard deviation.

*Table 2. Mitotic index (M.I.) of JB-1 ascites-tumor cells following administration of Colcemid 1 mg/kg at time zero. Signatures as in Fig. 2*

| | | No. of animals | Mean value of MI±S.D. 0 hr | ½ hr | 1½ hr | 2½ hr | 3½ hr | 4½ hr | 5½ hr |
|---|---|---|---|---|---|---|---|---|---|
| Exp. 1* | ●—● | 3 | 0·7±0·4 | 0·7±0·4 | 0·7±0·9 | 0·5±0·1 | 0·7±0·6 | 0·7±0·1 | 0·8±0·1 |
| | ○—○ | 3 | 0·8±0·1 | 0·8±0·1 | 1·2±0·1 | 1·8±0·4 | 2·1±0·4 | 2·0±0·1 | 2·4±0·3 |
| | ■—■ | 4 | 2·0±0·3 | 1·8±0·1 | 2·1±0·3 | 2·2±0·1 | 2·5±0·1 | 3·3±0·8 | 5·2±0·7 |
| | □—□ | 2 | 1·8—1·5 | 1·4—1·2 | 2·5—1·8 | 2·3—2·3 | 3·2—2·4 | 3·6—4·4 | 5·0—7·8 |
| | | | 0 hr | ½ hr | 1 hr | 2 hr | 3 hr | 4 hr | |
| Exp. II | ○—○ | 3 | 1·2±0·1 | 1·2±0·1 | 1·5±0·1 | 1·6±0·1 | 1·8±0·2 | 2·9±0·5 | |
| | ■—■ | 3 | 2·1±0·1 | 2·7±0·3 | 2·9±0·4 | 2·9±0·5 | 4·4±0·6 | 7·1±1·4 | |
| | □—□ | 3 | 2·1±0·1 | 2·2±0·1 | 2·5±0·1 | 2·6±0·1 | 3·2±0·3 | 5·9±0·8 | |

S.D.=Standard deviation.
*Fig. 1 represents the results of Exp. I.

growth of the controls was very slow and that the application of 1 ml of the tumor-ascitic fluid twice daily was insufficient to suppress the M.I. to the control level, which was probably a question of quantitation. However, it is possible that the inhibiting effect is very short lived so that a higher degree of mitotic suppression could be obtained with three or more daily applications of the tumor-ascitic fluid instead of two as used in this investigation.

Half of the ascitic tumor was removed 10 days after the inoculation leaving a considerable number of tumor cells and about 4 ml of tumor-ascitic fluid. A marked increase of the M.I. was seen (Fig. 3) which means that most of the inhibiting effect of the tumor ascitic fluid left behind had disappeared in 24 hr.

Injection of the tumor-ascitic fluid for only two days after the aspiration was followed by an increase of the M.I. This demonstrates that the tumor cells were still able to accelerate the growth following discontinuation of the inhibiting tumor-ascitic fluid (Fig. 4).

As demonstrated by Holmberg [10] the tumor-ascitic fluid might contain some cytotoxic products, but the viability of the JB-1 cells maintained in tissue culture and determined by Lissamine Green was not changed following application of the tumor ascites. The viability of the tumor cells on the tenth day after the inoculation was not significantly different from the viability two or three days after the inoculation.

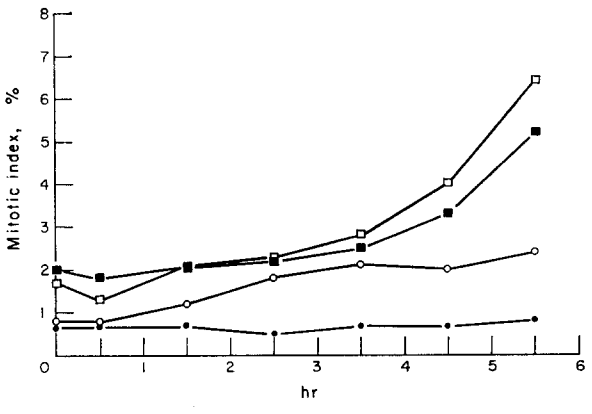


*Fig. 2. Mitotic counts in Colcemid (R)-treated mice 2 days after the aspiration. Signatures as in Fig. 1. Aspiration and subsequent injection of "the normal ascites" is indicated by* □—□.

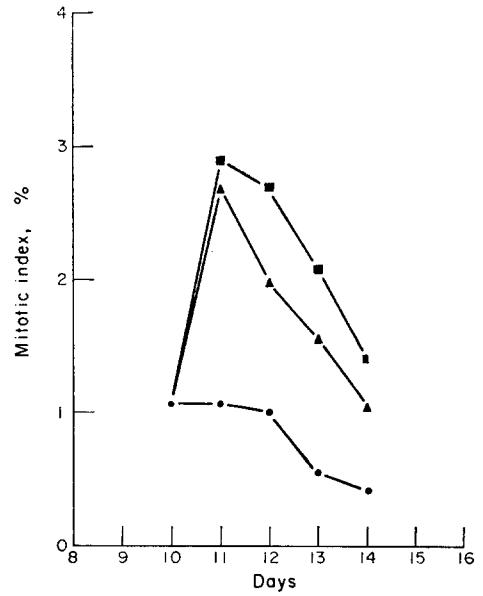


*Fig. 3. Mitotic counts of the controls (non-aspirated mice)* ●—●, *of the mice subjected to maximal aspiration* ■—■, *and the mice subjected to 50% aspiration* ▲—▲.

*Table 3. Mitotic index (M.I.) of JB-1 ascites-tumor cells from mice subjected to maximal aspiration (■—■), and mice from which only half of the ascitic tumor was removed (▲—▲)*

| | No. of animals | Mean value of M.I.±S.D. 1 day after aspiration | 2 days after aspiration | 3 days after aspiration | 4 days after aspiration |
|---|---|---|---|---|---|
| ■—■ | 13 | 2·9±0·6 | 2·7±0·4 | 2·1±0·4 | 1·4±0·3 |
| ▲—▲ | 9 | 2·7±0·6 | 2·0±0·5 | 1·6±0·4 | 1·1±0·2 |

M.I.=Mitotic index.
S.D.=Standard deviation.

*Table 4. Mitotic index (M.I.) of JB-1 ascites-tumor cells subjected to aspiration and subsequent injection twice daily of 1 ml of cell free tumor ascites for two days (I) and for three days (II)*

| | No. of animals | Mean value of M.I.±S.D. 1 day after aspiration | 2 days after aspiration | 3 days after aspiration | 4 days after aspiration |
|---|---|---|---|---|---|
| I* | 2 | 0·9–1·6 | 2·1–2·1 | 2·3 | 2·0 |
| II | 5 | 2·0±0·1 | 1·2±0·1 | 2·7±0·3 | 3·0±0·1 |

S.D.=Standard deviation.
*See Fig. 4.

## DISCUSSION

A negative feed back model of the mitotic control of the normal tissues has been suggested by several authors [11–13].

This theory was supported by the finding of the chalones [14, 15] which are tissue-specific but species-unspecific [16] mitotic inhibitors produced by the mature cells and influencing the progenitors of the same cells in order to maintain the equilibrium state of the tissue.

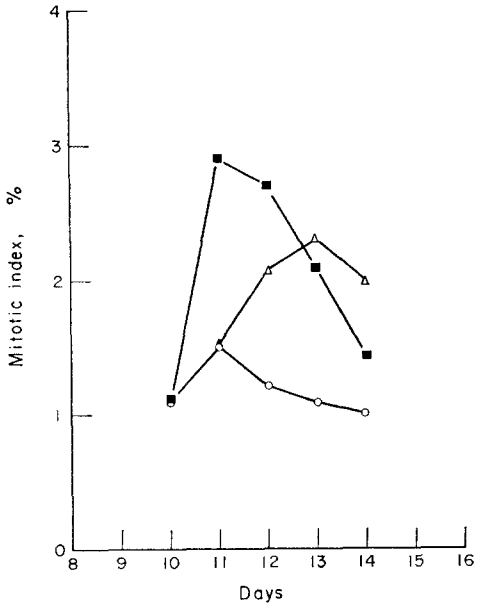


*Fig. 4. Mitotic counts of the mice subjected to maximal aspiration ■—■, the mice subjected to maximal aspiration and subsequent injection of the tumor ascitic fluid* O—O, *and maximal aspiration followed by injection of the tumor ascitic fluid for only two days after the aspiration* Δ—Δ.

The ascitic-tumor system offers several advantages for the investigation of the malignant growth regulation. The composition of the tumor is homogenous which means that even a small sample of the tumor will be representative for the total tumor, and it is likely that the phenomenon of contact inhibition plays no role because of the suspended condition of the ascitic tumor cells.

As mentioned above many tumors show a decrease in growth rate as the tumor increases in size, but as a rule they will kill the host before the equilibrium state is reached.

It is a characteristic feature of the tumor presented in this paper as well as for many other ascitic tumors that it attains a maximum level of tumor mass while the general condition of the animal is still acceptable.

Assuming an inverse relationship between the concentration of inhibitor and the proportion of cells in the generative cycle, the relation between the mitotic index and the population size will show a rapid initial rise followed by an exponential decay [17].

This corresponds very well to the course of the M.I. of the tumor in this paper and to the mathematical analysis of the growth of the

Ehrlich ascites tumor which is an exponential process limited by an exponential retardation [2], which is best described by a Gompertzian function.

Burns [7] showed that although the large, slow-growing Ehrlich ascites tumor at the plateau had almost stopped the incorporation of tritiated thymidine in DNA, the tumor cells continued synthesizing RNA and protein. This indicates that the decreasing growth rate hardly can be explained by a deficient nutrient supply.

The findings in this paper are in no way contradictory to recent results from the field of chalone-research. Bullough and Laurence studied a rabbit epidermal tumor Vx2 [19] and melanomas in mice and hamsters [20], and found that the tumor cells in these very different tumors contained the chalones, although reduced to about 1/10 of normal. Furthermore, the mitotic rate of the tumor showed a considerable response following application of their normal tissue specific chalones. When a large epidermal tumor was present, the content of chalone in the body was increased, and the mitotic rate of the normal epidermis was deeply depressed.

Regression of the tumors was seen when animals bearing melanomas were subjected to repeated injections of large amounts of skin extracts from pigs, which were known to contain the melanocytic chalone [21].

Rytömaa [15] investigated a rat granulocytic leukemia and found that the chalones from normal mature granulocytes and the leukemic cells were active on both the leukemic cells and the normal blood marrow cells. The leukemic cells proved to contain 1/10–1/40 the amount of chalone present in the normal mature granulocytes and excess of chalone was found in the leukemic serum. The normal blood marrow cells were significantly more sensitive to the chalone than were the leukemic cells.

Although the content of chalone in the body of tumor-bearing animals was raised, the concentration was insufficient to prevent a progressive growth of the tumor. We might explain these findings by the fact that the tumors were investigated at a time when they were systems under development, which means situations characterized by instability. Probably most normal organs under development would reveal similar features of growth regulation.

Although other mechanisms must be taken into consideration the self-inhibition of the ascites tumor cells by means of a humoral mitotic inhibitor might be one of the explanations of the growth regulation in this tumor system.

At present the relation between the mitotic inhibitor described in this paper and the chalones is not clear. Work is in progress to investigate the specificity and the physical and chemical characteristics of this inhibiting principle.

## SUMMARY

*Reduction of an ascites tumor JB-1 was followed by a second increase of the growth rate. Inhibition of this recurrent growth was observed following daily applications of the cell free tumor ascites obtained from mice bearing JB-1 ascites tumors at the plateau phase of growth.*

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# Pharmaco-biochemical Studies on Cytotoxic Polyol Derivatives—II

## The Effect of Biological Alkylating Agents on the Thermal-Denaturation Properties of DNA

A. JENEY, Jr.,* I. SZABÓ, T. VÁLYI-NAGY,† L. INSTITORIS and J. SZABÓ

*Department of Pharmacology, University Medical School of Debrecen and Chinoin Factory for Pharmaceutical and Chemical Products, Budapest, Hungary*

### INTRODUCTION

IT WAS reported in our previous paper [1] that dibromodulcitol—a potent inhibitor of malignant cell growth—does not influence the thermal-denaturation profile of DNA. Since dibromodulcitol is a potential alkylating agent, we compared the effect of certain cytotoxic polyol derivatives with some nitrogen mustards and dimethylmyleran on the thermal-denaturation properties of DNA.

A study of the thermal-denaturation profile of DNA offers a suitable model for measuring the direct effect of drugs on that particular macromolecule, since other constituents of the cell would not be present in this *in vitro* system. The only drawback of the method is that a high concentration of the drug surpassing the pharmacological dose level is needed. In addition Wheeler *et al.* [2] have found an alteration of the thermal-denaturation profile of DNA only after *in vitro*, but not *in vivo* treatment with the biological alkylating agents.

Histones are able to increase the Tm point of the DNA [3]. This phenomenon renders it possible to study the interaction between histone and DNA in the presence of drugs. Since it was found that dibromohexitols enhance the incorporation of $C^{14}$-orotic acid in certain RNA [1], we assumed that this is mediated through an altered function of the histones. To test this hypothesis, the Tm point of DNA was measured in the presence of histones treated *in vitro* with the biological alkylating agents.

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*Present address: Christie Hospital and Holt Radium Institute, Manchester 20.

†Before finishing this paper Prof. T. Vályi-Nagy died, whom we greatly miss in further enlarging on this project.

### MATERIAL AND METHODS

Calf-thymus DNA was prepared according to the method of Colter *et al.* [4]. The analytical data for this DNA are as follows: $P\%$: 9·17, $N\%$: 13·90. $\lambda$ maximum: 257 m$\mu$, $E280/E260$: 0·533, $\varepsilon$ ($P$): 6·500, increasing in absorption by: 47·7% (after acidic hydrolysis) and 31·4% (after DNAase hydrolysis). Base composition (mol %:) guanine: 21·7, cytosine: 20·9, adenine: 27·2, thymine: 30·2.

The DNA was dissolved in a concentration of 12·5 $\mu$g/ml in 0·1 SSC buffer (0·015 M NaCl+0·0015 M Na-citrate, pH 7·1) or in a lower ionic strength buffer (2·8 m$M$ NaCl+0·2 m$M$ Na-citrate, pH 6·6). The DNA was preincubated with the appropriate concentration of the drug for two hours at 25°C. The cuvettes containing the control or the drug-treated DNA were placed in a modified "Spectromom" spectrophotometer equipped with a thermal spacer connected to a temperature controlled circulating water bath. The

| | Drug | Tm | ΔTm | Hyperchromic effect in % | ΔHyperchrom |
|---|---|---|---|---|---|
| 1 | DNA control | 62·5 | — | 40·0 | — |
| 2 | Degranol | 53·5 | −9·0 | 38·5 | −1·5 |
| 3 | Nitrogenmustard | 52·5 | −10·0 | 33·5 | −6·5 |
| 4 | Merophan | 55·0 | −7·5 | 26·8 | −13·2 |
| 5 | Endoxan | 56·0 | −6·5 | 29·4 | −10·6 |
| 6 | Dimethylmyleran | 63·0 | 0·5 | 17·0 | −23·0 |

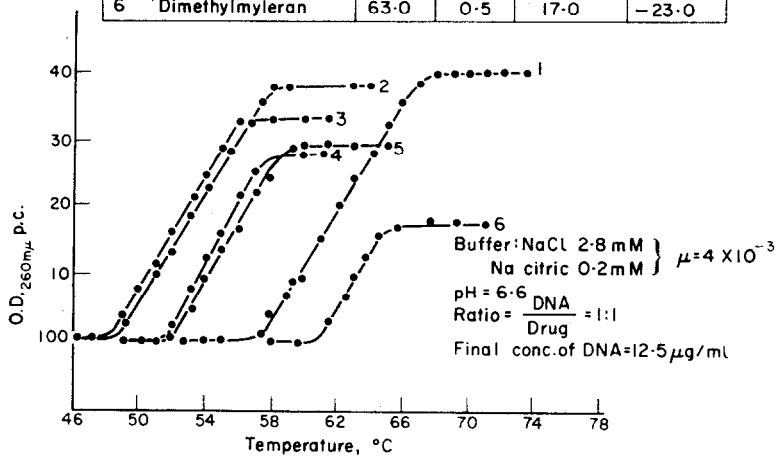



*Fig.* 1. *The effect of some biological alkylating agents on the thermal-denaturation profile of thymus DNA. Abscissa: temperature (°C). Ordinate: relative absorbance at* 260 mμ.

temperature in the cuvettes was checked by a thermistor, and raised slowly (1° in 5 min), and the optical density was read at 260μ.

For measuring the effect of histone on the DNA, the histone was dissolved in 2·8 m*M* NaCl–0·2 m*M* Na-citrate, pH 6·6 buffer, and pretreated with the drug for two hours at room temperature. The histone was added to the DNA dropwise, which was also dissolved in this low ionic strength buffer. The ratio between the DNA and the histone was 1:1 (12·5 μg) ml.

The unfractionated histone used in our experiments was prepared by Dr E. W. Johns (Chester Beatty Research Institute, London) and kindly placed at our disposal. Degranol, dibromomannitol, dibromodulcitol, dichloromannitol and mannitolmyleran were produced by Chinoin Factory for Chemical and Pharmaceutical Products. Nitrogen mustard, Merophan and dimethylmyleran are the generous gift of Dr T. A. Connors (Chester Beatty Research Institute, London). Endoxan was purchased from the Asta-Werke (Germany).

## EXPERIMENTS

*The direct effect of drugs on the thermal-denaturation profile of DNA*

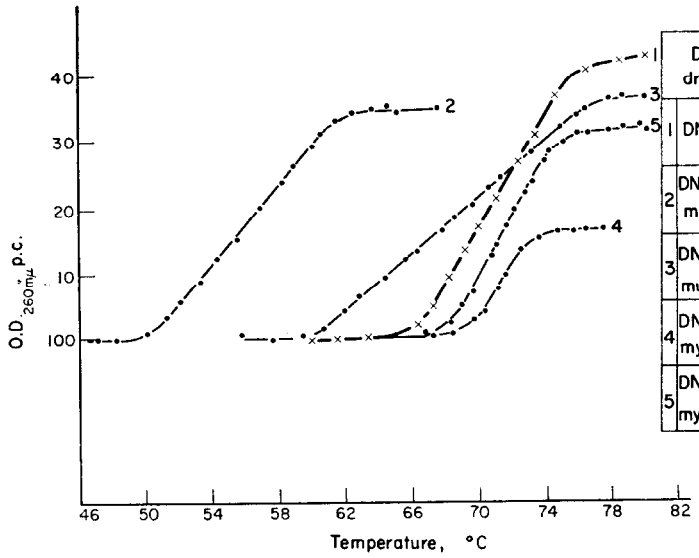
Figure 1 shows that the Tm point of untreated DNA is 62·5°C at the ionic strength employed by us. Using equivalent concentrations of DNA and the alkylating agents, the thermal-denaturation property of DNA was influenced differentially. Nitrogen mustard produced only a slight hyperchromic shift in the DNA, whereas it reduced the Tm point by about 10 deg. On treatment with Endoxan and Merophan the Tm point of DNA was not reduced to such an extent, but the hyperchromic effect was considerably affected. In the presence of dimethylmyleran the rate of the temperature absorption increase of DNA lagged behind that of the control, but scarcely differed from its starting temperature.

We investigated how the alterations of the thermal-denaturation curve of DNA produced by nitrogen mustard and dimethylmyleran was modified when lower concentrations were used. It can be seen in Fig. 2 that the lower concentrations of the two alkylating agents changed the thermal-denaturation curve of the thymus DNA proportionately less but qualitatively similar to that observed at a higher concentration.

Of the cytotoxic polyol derivatives examined only Degranol—which possesses the chloroethylamino group—influenced the thermal denaturation property of DNA (Fig. 3). Degranol, like nitrogen mustard, did not change the hyperchromicity of DNA but the Tm point is decreased by 14·5°C. Dibromodulcitol did not affect the thermal-denaturation property of DNA even when the incubation temperature, the time or the pH of the medium was changed.

*The effect of drugs on the interaction of histone and DNA*

It was then investigated whether those cytotoxic polyol derivatives which do not affect the thermal-denaturation property of DNA, changed the effect of histone on the Tm point of DNA. Figure 4 shows that the control histone raised the Tm point of DNA by 10°C. But histone pretreated with dibromodulcitol and mannitolmyleran lost its ability to affect the Tm point of DNA. Histone pretreated with dibromomannitol raised the Tm point of DNA by only 6°C. The chemotherapeutic significance of the effect was shown by the fact that dichloromannitol, which is chemically closely related to the dibromohexitols but which does not possess any tumour-inhibiting effect, did not influence the effect of histone on the Tm point of DNA. The above-mentioned effects remained unchanged even in the case of histone




| | DNA and drug ratio | Tm | ΔTm | Hyperchrom effect in % | ΔHyper-chrom |
|---|---|---|---|---|---|
| 1 | DNA control | 70·0 | – | 41·0 | – |
| 2 | DNA+nitrogen-mustard 1:1 | 55·5 | −14·5 | 33·0 | −8·0 |
| 3 | DNA+nitrogen-mustard 1:0·1 | 66·5 | −3·5 | 36·8 | −4·2 |
| 4 | DNA+dimethyl-myleran 1:1 | 69·5 | −0·5 | 15·0 | −26·0 |
| 5 | DNA+dimethyl-myleran 1:0·1 | 71·0 | 1·0 | 32·0 | −9·0 |

*Fig.* 2. *The effect of various concentrations of nitrogen mustard and dimethylmyleran on the thermal-denaturation profile of DNA.*
*Abscissa: temperature* (°C).
*Ordinate: relative absorbance at* 260 mμ.
*Thymus DNA and the drugs were dissolved in* 0·1 *SSC buffer* (0·15 M *NaCl*+0·0015 M *Na-citrate*, *pH* 7·1).

| Drug | Tm | ΔTm | Hyperchromic effect in % | ΔHyper-chrom |
|---|---|---|---|---|
| 1 ••• DNA alone | 70·0 | – | 40·5 | – |
| 2 — — Degranol | 55·5 | −14·5 | 39·0 | − 1·5 |
| 3 ××× Dibromodulcitol DBD | 70·0 | ϕ | 40·2 | − 0·3 |
| 4 ooo Dimesylmannitol DMM | 69·7 | −0·3 | 40·5 | ϕ |
| 5 ΔΔΔ Dibromomannitol DBM | 70·0 | ϕ | 40·5 | ϕ |
| 6 ooo Dichloromannitol DCM | 70·0 | ϕ | 40·5 | ϕ |

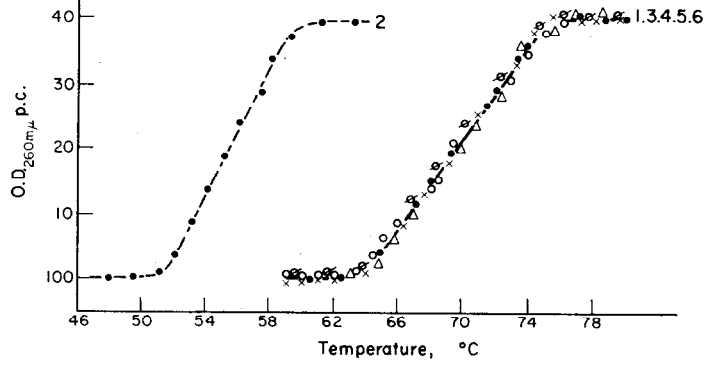



*Fig.* 3. *The effect of cytotoxic hexitols on the thermal-denaturation profile of thymus DNA.*
*Abscissa: temperature* (°C).
*Ordinate: relative absorbance at* 260 mμ.

pretreated with cytotoxic hexitols which had been dialysed against a hundredfold volume of buffer to remove the unbound drug.

The effect of histone on DNA was influenced by cytotoxic hexitols only in the case of histone was treated with agents before being added to DNA. If dibromodulcitol or mannitolmyleran were added to the already preformed nucleohistone, no change was observed in the thermal-denaturation curve (Fig. 5).

The investigation of the effect of the examined mustard derivatives and dimethylmyleran on histone is rendered difficult by the fact that each of them can change the thermal denaturation property of DNA by itself. In our experiments we wished to gain an answer to the question of the modification of the thermal denaturation curve of DNA by incubation with histone and, on the other hand, whether they influence the effect of histone. This is all the more difficult to decide in the case of nitrogen mustard and Degranol, because they alter the hyperchromicity of DNA slightly, while at the same time they

| Drug | Tm | ΔTm | Hyperchromic effect in % | ΔHyperchrom |
|---|---|---|---|---|
| 1 ••• DNA alone | 63·00 | — | 40·8 | — |
| 2 ××× DNA + (H + DBD) | 63·50 | −9·50 | 40·0 | − 0·2 |
| 3 ooo DNA + (H + DMM) | 63·25 | −9·75 | 40·5 | 0·3 |
| 4 ΔΔΔ DNA + (H + DBM) | 69·50 | −3·50 | 40·0 | − 0·2 |
| 5 □□□ DNA + histone (H) | 73·00 | — | 40·2 | — |
| 6 ⌀⌀⌀ DNA + (H + DCM) | 73·50 | +0·50 | 39·6 | −0·6 |

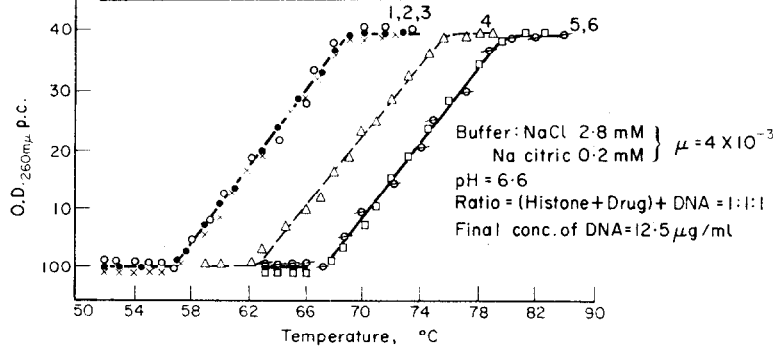



*Fig. 4. The effect of cytotoxic hexitols on the formation of the DNA-histone complex.*
*Abscissa: temperature (°C).*
*Ordinate: relative absorbance at 260 mμ.*
*Abbreviations: H ± histone, DBD ± dibromodulcitol, DMM ± mannitomyleran, DBM ± dibromomannitol, DCM ± dichloromannitol.*

| Drug | Tm °C | Δ Tm | Hyperchromic effect in % | ΔHyperchrom |
|---|---|---|---|---|
| 1 — — — DNA alone | 62 | — | 40 | — |
| 2 ••• DNA + histone (H) | 72 | — | 40 | — |
| 3 ××× (DNA + H) + DBD | 72 | ϕ | 40 | ϕ |
| 4 ooo (DNA + H) + DMM | 72 | ϕ | 40 | ϕ |

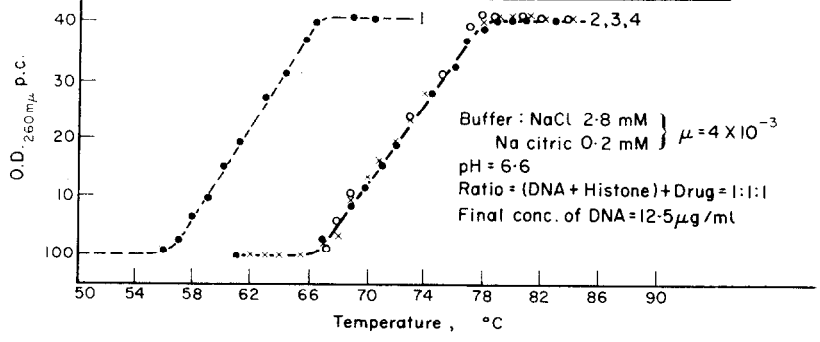
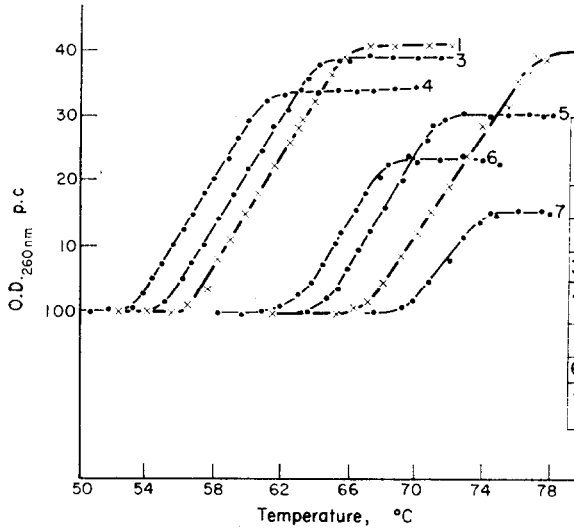



*Fig. 5. The effect of cytotoxic hexitols on the preformed DNA-histone complex.*
*Abscissa: temperature (°C).*
*Ordinate: relative absorbance at 260 mμ.*
*Abbreviations: H = histone, DBD = dibromodulcitol. DMM = mannitomyleran.*




| | Drug | Tm | ΔTm | Hyperchromic effect in % | ΔHyperchrom |
|---|---|---|---|---|---|
| 1 | DNA alone | 63·0 | - | 40·6 | — |
| 2 | DNA+histone(H) | 73·0 | 10·0 | 40·3 | −0·3 |
| 3 | DNA+(H+degranol) | 60·0 | −3·0 | 39·5 | −1·1 |
| 4 | DNA+(H+nitrogenmustard | 58·5 | −4·5 | 35·0 | −5·6 |
| 5 | DNA+(H+endoxan) | 68·5 | 5·5 | 30·2 | −10·4 |
| 6 | DNA+(H+merophan) | 66·0 | 3·0 | 22·6 | −18·0 |
| 7 | DNA+(H+dymethylmyleran) | 71·5 | 8·5 | 15·6 | −25·0 |

*Fig. 6. The effect of some biological alkylating agents on the formation of the DNA-histone complex.*
*Abscissa: temperature (°C).*
*Ordinate: relative absorbance at 260 mμ.*
*Thymus DNA and the drugs were dissolved in 2·8 mM NaCl+0·2 Na citrate, pH 6·6 buffer.*

decrease the Tm point to approximately the same degree as the histone increases it. For this reason it cannot be established from this pattern whether Degranol and nitrogen mustard suspend the effect of histone, or in spite of the nucleohistone complex formed, its Tm point is decreased by damaging the DNA. Endoxan, Merophan and dimethylmyleran however, influence also the hyperchromicity of DNA which does not change in the presence of histone. It is noteworthy that Endoxan, Merophan and dimethylmyleran—after pre-incubation with histone—decrease the hyperchromic effect to the same extent as without histone (Fig. 6) [1]. It appears that in these latter cases a nucleohistone complex is formed because the Tm point of DNA increases in spite of the fact that the chemical agents have decreased the hyperchromicity.

## DISCUSSION

The elaboration of the temperature denaturation curve of DNA into an exact method is the merit of Marmur and Doty [5–7].

Using this method Kit [8] was unable to find any difference between the thermal-denaturation curve of DNA of certain experimental tumours and that of normal tissues. Brewer *et al.* [9] and later Wheeler *et al.* [2] observed a decrease of the Tm point of DNA influenced *in vitro* with nitrogen mustard at a concentration of $10^{-4}$ and $10^{-5}M$. From this they concluded a significant weakening of the stability of the double helix.

The biological alkylating agents investigated by us can be classified on the basis of their effect on the thermal-denaturation property of DNA or nucleohistone respectively. The aliphatic and aromatic nitrogen mustards, as well as dimethylmyleran altered the thermal-denaturation profile of DNA very profoundly, which indicates its impairment. We consider it striking that the alkylating agents shown in Fig. 1 influence the Tm point of DNA in an inverse relationship to the hyperchromicity, the latter expressing the rate of separation of the polynucleotide chain. When evaluating this experiment, it may be assumed that the cross-linkage of the two polynucleotide chains of the double helix are brought about most markedly by dimethylmyleran, because this agent influenced the hyperchromicity the most strongly. If DNA is treated with nitrogen mustard or Degranol, its absorption began to rise at a lower temperature, leading us to suppose that the alkylation took place on one of the polynucleotide chains, as a result of which, the DNA got into a state of assymetrical vibration.

The tumour-inhibiting effect of the dibromohexitols and mannitolmyleran by far surpasses that of the biological alkylating agents investigated in the foregoing. Hence it is all the more striking that they do not influence the thermal-denaturation property of DNA. Of course, it cannot be excluded that these agents damage DNA only on the basis of this one method. From the character of their effect on nucleohistone it may be assumed that the dibromohexitols and mannitolmyleran alkyl-

ate the sites of DNA which form ionic bonds with the basic groups of the histones. This supposition, however, we do not consider probable, because the alkylation of the phosphate esters would lead to the alteration of the thermal-denaturation profile of DNA.

Since histone, even after dialysis, is unable to raise the Tm point of DNA, we are inclined to suppose that the dibromohexitols and mannitolmyleran prevent the formation of the nucleohistone complex by inhibiting the function of histone. This experiment suggests at the same time that these agents are bound to histone, or their effects are irreversible. Considering that they are incapable of changing the Tm point of the already preformed nucleohistone, we are of the opinion that they are able to form bonds only with free histone. It may be assumed that they are capable of suspending the repressor effect of the histones on the template activity of DNA, which may be correlated with the increased RNA synthesis following dibromohexitols [1]. It is possible that the discriminating action of dibromohexitols and mannitolmyleran on histone and DNA respectively, is one of the biochemical manifestations of the selective tumour-inhibiting effects they exhibit.

**Acknowledgement**—We would like to thank Dr B. W. Fox for his assistance in the final stages of this manuscript.

## SUMMARY

*The effect of the biological alkylating agents on the thermal-denaturation profile of DNA and on nucleohistone was investigated. It was established that while the mustard derivatives and dimethylmyleran profoundly, though differentially, altered the thermal-denaturation property of DNA, the dibromohexitols and mannitolmyleran proved to be ineffective. On the other hand the dibromohexitols and mannitolmyleran are capable to blocking the effect of histone on the Tm point of DNA.*

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# 4'-Demethyl-epipodophyllotoxin Thenylidene Glucoside (VM 26), a Podophyllum Compound with a New Mechanism of Action

H. STÄHELIN
*Biological and Medical Research Division, Sandoz Ltd., Basel, Switzerland*

## INTRODUCTION

AFTER Kaplan [1] had discovered the curing effect of podophyllin in the treatment of condylomata acuminata, Sullivan and Wechsler [2], Cornman [3] and others described arrest in metaphase produced by this plant extract. The mechanism of action thus seemed to be the same as that of another plant product: colchicine. The main active constituent of podophyllin is podophyllotoxin [4]. This latter compound is a typical spindle poison and its effects on dividing plant or animal cells are morphologically very similar to those of colchicine [5]. Further chemical and biological investigations with podophyllum compounds [6, 7] led to the development of two preparations [8] now in clinical use for the treatment of solid tumors [9]: SP-I (podophyllic acid ethyl hydrazide) and SP-G (mixture of purified benzylidene glucosides of podophyllum emodi). Both drugs arrest mitosis in metaphase, they are spindle poisons.

Newer developments in our research laboratories led to a podophyllotoxin glucoside derivative which had a high cytostatic activity in cell cultures but which, contrary to all previously known active podophyllotoxin derivatives, did not produce accumulation of metaphases. Some of the biological effects of this compound, 4'-demethyl-epipodophyllotoxin thenylidene glucoside (or: 4'-demethyl-1-0-4, 6-0-thenylidene-β-D-glucopyranosyl) epipodophyllotoxin), which is now under clinical trial as VM 26, shall be described in the following. A few of the results have been presented previously in a short communication [10].

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## METHODS

1. *Dissolution of the drug*

Since VM 26 is poorly water soluble, organic solvents had to be used. Clear aqueous solutions were obtained by dissolving the drug powder in dimethyl sulfoxide, adding Tween 80 and then water. The concentrations of the two solvents were kept at a level so as not to produce toxicity *in vitro* or *in vivo*. Effects of DMSO or Tween on the parameters measured were excluded by control experiments and by always treating the control animals with water containing DMSO and Tween in the concentrations used for the treated animals.

2. *Cell cultures*

P-815 mastocytoma and KB cells were cultured and used for quantitative evaluation of cytostatic activity *in vitro* as described previously [11].

Cultures of chick embryo fibroblasts were set up and evaluated, after addition of VM 26, as outlined previously [7, 8].

3. *Animals*

The animals used in different experiments are indicated in the text.

SIM=Swiss mice kept in a closed colony for many years in our animal farm. DBA/2 were originally obtained from Jackson laboratories (Bar Harbor, Me.) and propagated in our animal farm. Mice were used when they were 8–10 weeks old. SDF=(SIM×DBA/2) $F_1$ mice.

Rats: Wistar rats, obtained from Meyer-Arend (Bad-Salzuflen, Germany), were used when their weight was 150–200 g.

Since no influence of sex of the animals on the results was detected, this factor is not mentioned below.

4. *Tumors*

Oncostatic activity of VM 26 in animals was evaluated with 6 mouse tumors and 1 rat tumor. Sarcomas 37, 180, and Walker carcinosarcoma were inoculated s.c. into the flank of the animals. Leukemias P-815 and L-1210 were inoculated subcutaneously into the hind leg (if not otherwise indicated), leukemia P-1534 was implanted subcutaneously into the flank of the mice. Inoculum was ascitic fluid for all tumors except P-1534; in this tumor, pieces of subcutaneously growing tumor were implanted by trocar.

In every experiment, 5 groups of 6 animals each were used; one control group received the solvent, the other groups were treated with 4 logarithmically spaced doses of VM 26. The first day of treatment and treatment schedules are indicated in Tables 5 and 6. The total number of injections per animal was the following if not indicated in Tables 5 and 6: for daily treatment (except Sundays) and evaluation by tumor weights, 6; daily treatment, and evaluation by survival time till death of the animal, but not more than 12; twice weekly, and evaluation by survival time till death of the animal, but not more than 6. Evaluation was either by tumor weight or by survival time; in the Ehrlich ascites, tumor weight was replaced by total packed cell volume. The antitumor effect is given in per cent, either as reduction of tumor weight (compared to controls) or as increase in life span (ILS) over that of controls; survival time is the number of days elapsing from implantation of the tumor to the death of the animal. Survivors were, of course, not included in the calculation of ILS.

## RESULTS

1. *Activity in cell cultures*

(*a*) *Inhibition of cell multiplication.* Cytostatic activity was evaluated quantitatively in cultures of a permanent line of tumor cells. Concentrations of VM 26 and, for comparison of other drugs, inhibiting proliferation of P-815 cells by 50% (ED-50) are given in Table 1. ED-50 of VM 26 for L cells and KB cells is somewhat higher than for P-815 cells, 0·008 and 0·012 mg/l respectively.

*Table* 1. *Inhibition of proliferation of P-815 mastocytoma cells* in vitro

| Drug | ED-50 (mg/l) |
|---|---|
| VM 26 | 0·005 |
| podophyllotoxin | 0·005 |
| colchicine | 0·004 |
| demecolcine | 0·014 |
| vinblastine | 0·008 |
| amethopterin | 0·010 |
| 6-mercaptopurine | 0·29 |
| mechlorethamine (HN2) | 0·02 |
| verrucarin A | 0·0005 |

(*b*) *Effects on fibroblasts* in vitro. In order to evaluate the morphological alterations which are caused by the active substance in resting and dividing animal cells, pieces of connective tissue of chick embryos are cultured *in vitro*. The fibroblasts migrating from the explant show a high rate of cell division and are spread out in a flat sheet so that resting cells as well as mitoses can easily be observed. The test substance is added in different concentrations for some hours to the cultures; afterwards the preparations are fixed and stained with hemalum. The different phases of mitosis per 1000 cells are counted (mitotic index) and the morphological changes of the resting and dividing cells are registered.

The results of the mitotic counts are given in Table 2; in these figures, the cells in the process of lysis are not included.

*Table* 2. *Mitotic counts in fibroblast cultures* 3 *hr after addition of VM* 26. *The number of pro-, meta-, and ana-telophases is given per* 1000 *cells*

| Concentration VM 26 mg/l | prophases | metaphases | ana- and telophases |
|---|---|---|---|
| 0 | 13 | 18 | 10 |
| 0·1 | 7 | 11 | 6 |
| 0·32 | 0·5 | 2 | 0·5 |
| 1·0 | 0 | 0 | 0 |

The first effect of VM 26 is to arrest mitosis at metaphase, thus the compound seems to have the properties of a spindle poison. After 30–60 min, however, this effect is gradually superseded by another effect, i.e. lysis of cells

*Table* 3. *Reversibility of the antimitotic effect of VM* 26 *and spindle poisons in fibroblast cultures*

| Drug | Concentration mg/l | Hours without drug ½ | 1 | 2 | 4 | 7 | No. of exp. |
|---|---|---|---|---|---|---|---|
| VM 26 | 1·0 | — | 0 | — | 0·16 | 0·18 | 2 |
| Podophyllotoxin | 0·01 | 0 | 0·20 | 1·0 | — | 1·0 | 1 |
| SPI | 3·2 | 0·25 | 1·1 | 0·71 | 0·98 | 0·86 | 4 |
| Demecolcine | 0·1 | 0 | 0·23 | 0·80 | 0·70 | 0·77 | 2 |
| Vinblastine | 0·01 | 0 | — | 0·32 | 1·1 | 0·83 | 2 |

Cultures were treated with drug 2 hr, then washed and incubated without drug. Figures are relative numbers (control = 1·0) of ana- and telophases per 1000 cells. In each experiment, 4 treated cultures were counted.

(at concentrations of 1 mg/l or more, Figs. 1–5) or inhibition of beginning of mitosis (at concentrations between 0·1 and 1 mg/l.). At the higher concentrations, cells preparing for mitosis disintegrate into small droplets; at the lower concentrations disintegration seems to become rare, but the number of all mitotic phases (including prophase) is zero or greatly diminished. The concentration necessary for a complete or a 70% inhibition of mitosis depends on the duration of the experiment; it decreases during the first few hours. Since VM 26 prevents cells from entering prophase or metaphase of mitosis, it is evident that the action on the mitotic spindle cannot become effective any more.

The effect of VM 26 in these fibroblast cultures is practically irreversible. If substance is added to the cultures for one hour and then washed out, the effect is (for at least 24 hr) the same as in those cultures where the substance is left in contact with the cells.

The irreversibility of the effect of VM 26 in fibroblast cultures is in contrast to the reversibility of the mitotic arrest elicited by podophyllum compounds of the pure spindle poison type and by colchicine, the classical spindle poison, as well as the metaphase block produced by vinblastine. When chick-embryo fibroblast cultures are treated with one of these drugs and the mitotic poison is washed away after some time, mitoses reappear in a normal number within a few hours. This is not so with VM 26 where only few ana- and telophases (as a parameter of cell proliferation) reappear within 7 hr after removal of the drug (Table 3). The concentrations indicated in Table 3 are the minimal concentrations which produce consistently total mitotic inhibition (no ana- or telophases among 1000 or more cells) within 2 hr in chick embryo fibroblast cultures. The number of pro and early metaphases is not changed by treatment with pure spindle poisons, in contrast to the cultures to which VM 26 has been added.

2. *Toxicity*

Doses of VM 26 killing one out of six animals (LD-17) were determined in SDF mice and in Wistar rats. The drug is given either once only or daily for 6 days or twice weekly for 1½ (3 injections) or 2 (4 injections) weeks. The results are given in Table 4. They were calculated by the method of Reed and Muench [12].

*Table* 4. *Acute and subacute toxicity of VM* 26 *in SDF mice and in Wistar rats*

| Injections | LD-17, mg/kg/injection i.p. Mouse | Rat |
|---|---|---|
| Single | 51 | 52 |
| Daily, 6× | 5·3 | 6·0 |
| Daily, 12× | 5·0 | — |
| Twice weekly, 4× | 17 | — |
| Twice weekly, 3× | — | 9·0 |

Even with single i.p. doses around or higher than LD-50, mice and rats never died earlier than 3 days after injection of VM 26, occasional animals only on days 12–14. It should be noted from Table 4 that in mice equitoxic doses per week are larger when given in one or two injections than when given with 6 (daily) injections.

3. *Antitumor effect*

Tumor inhibition by VM 26 was tested in 7 transplantable rodent tumors using *ca.* 1750 mice and 300 rats. The test procedures are described under "Methods". The results with

*Table 5. Antitumor activity of VM 26 against 6 transplantable rodent tumors*

| Tumor | Host | Treatment: start day | Treatment: injections | Dose (mg/kg) | % anti-tumor activity | Survivors* | No. of exp. |
|---|---|---|---|---|---|---|---|
| Sarcoma 37 s.c. | SIM | 2 | daily | 1·0 i.p. | 41 | — | 2 |
| | SDF | 2 | daily | 4·0 i.v. | 51 | — | 1 |
| | SDF | 1 | day 1, 3, 7 | 4·0 i.v. | 68 | — | 1 |
| Sarcoma s.c. | SIM | 2 | daily | 2·0 i.p. | 42 | — | 1 |
| | SDF | 2 | daily | 2·0 i.p. | 51 | — | 1 |
| | SDF | 1 | day 1, 3, 7 | 6·0 i.p. | 72 | — | 2 |
| Ehrlich ascites i.p. | SIM | 2 | daily | 0·6 i.p. | 100 | — | 1 |
| | SIM | 2 | day 2, 6 | 3·0 i.p. | 100 | — | 1 |
| | SIM | 2 | 2×/week | 12 i.p. | 75 | 0 | 1 |
| Mastocytoma P-815, s.c. | SDF | 1 | daily | 1·0 i.p. | 30 | 0 | 1 |
| | SDF | 1 | 2×/week | 10 i.p. | 59 | 0 | 2 |
| Leukemia P-1534 s.c. | DBA/2 | 2 | 2×/week | 8 i.v. | 56 | 1 | 1 |
| Walker carcinosarcoma s.c. | Wistar rat | 2 | daily | 2·7 i.p. | 68 | — | 4 |
| | | 2 | daily | 4·0 i.v. | 86 | — | 1 |
| | | 1 | day 1, 3, 6 | 8·2 i.p. | 78 | — | 3 |
| | | 1 | day 1, 3, 6 | 8·0 i.v. | 96 | — | 1 |

*In this column, — indicates evaluation by tumor weight (animals were killed); a figure is there when antitumor activity was evaluated by survival time increase.

*Table 6. Increase in life span (ILS) by VM 26 in mice inoculated with leukemia L-1210*

| Inoculum (No. of cells) | Host | Treatment: start day | Treatment: injections | Dose (mg/kg) | ILS % | Survivors | No. of exp. |
|---|---|---|---|---|---|---|---|
| $10^6$ s.c. | SDF | 1 | daily | 2·6 i.p. | 121 | 0 | 7 |
| | | 1 | daily | 2·9 i.v. | 136 | 0 | 1 |
| | | 1 | daily | 2·9 s.c. | 100 | 0 | 1 |
| | | 1–2 | 2×/week | 17 i.p. | 165 | 0 | 3 |
| | | 1 | 2×/week | 11 i.v. | 172 | 0 | 3 |
| | | 5 | 2×/week | 10 i.p. | 172 | 1 | 2 |
| | | 1 | 1×/week | 32 i.p. | 65 | 0 | 1 |
| | | 1 | once | 36 i.p. | 126 | 0 | 2 |
| | | 2–4 | once | 33 i.p. | 121 | 2 | 4 |
| | | 5 or 7 | once | 54 i.p. | 57 | 0 | 2 |
| $10^6$ s.c. | DBA/2 | 1 | daily | 2·0 i.p. | 89 | 0 | 1 |
| | | 5 | daily | 2·0 i.p. | 54 | 0 | 1 |
| | | 1 | daily | 1·0 i.v. | 70 | 0 | 1 |
| | | 1 | 2×/week | 16 i.p. | 146 | 0 | 1 |
| $10^4$ s.c. | SDF | 2 | 2×/week | 12 i.p. | 151 | 4 | 1 |
| | | 1 | once | 18 i.p. | 94 | 4 | 2 |
| $10^3$ s.c. | SDF | 2 | 2×/week | 12 i.p. | 170 | 0 | 1 |
| | | 1 | once | 12 i.p. | 62 | 5 | 2 |
| $10^6$ i.p. | SDF | 1 | daily | 2·0 i.p. | 246 | 3 | 1 |
| | | 1 | 2×/week | 6·0 i.p. | 373 | 8 | 2* |
| $10^6$ i.p. | DBA/2 | 1 | daily | 4·0 i.p. | 286 | 0 | 1 |
| $10^4$ intracerebr. | SDF | 1 | daily | 4·0 i.p. | 36 | 0 | 1 |
| | | 2 | 2×/week | 9·0 i.p. | 47 | 0 | 2 |
| | | 3 | once | 40 i.p. | 56 | 0 | 1 |

*In these experiments, mice were treated 8 times (i.e. twice weekly for 4 weeks if they lived that long).

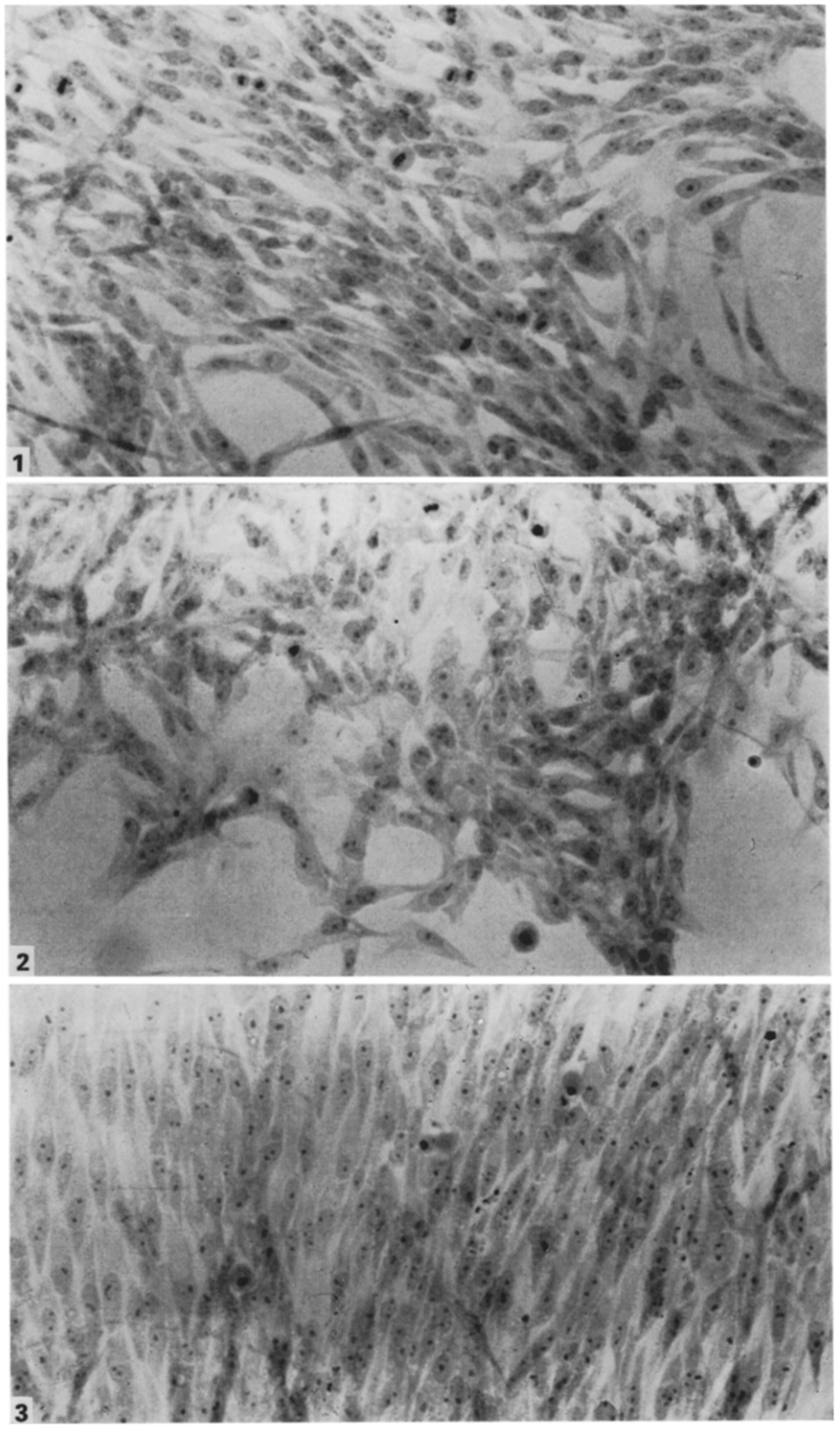


*Fig.* 1. *Untreated control. Mitotic cells in different stages.*

*Fig.* 2. *After* 30 *min incubation with VM* 26, 3·2 *mg/l. Blocked metaphases (c-mitoses) begin to appear, late phases (ana- and telophases) have disappeared.*

*Fig.* 3. *After* 1 *hr incubation with VM* 26, 3·2 *mg/l. Blocked metaphases (c-mitoses) still more characteristic than after* 30 *min. No late phases. Disintegrating cells begin to show up.*

(to face p. 306)

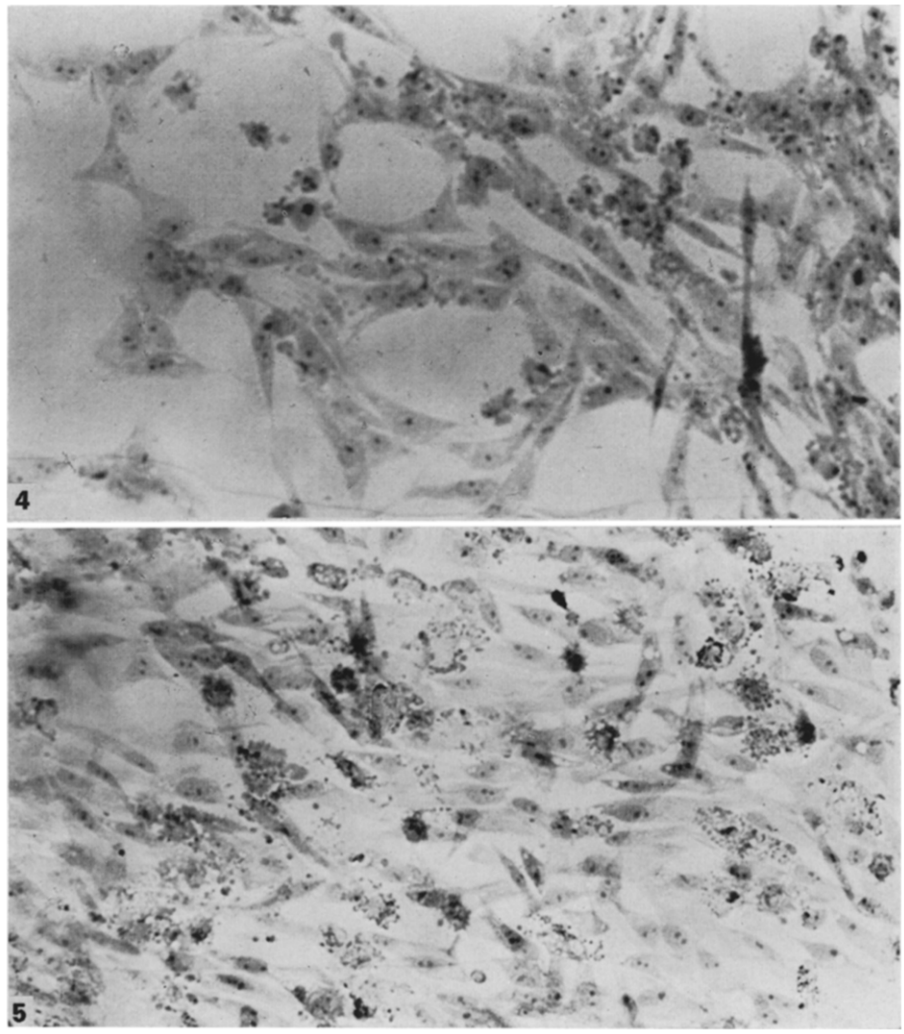


*Fig.* 4. *After* 2 *hr incubation with VM* 26, 3·2 *mg/l. Blocked metaphases have mostly disappeared. The number of disintegrating cells increases.*

*Fig.* 5. *After* 3 *hr incubation with VM* 26, 3·2 *mg/l. Many cells (those in the process of preparing for division) are disintegrating. Cells in interphase seem unaffected.*

further details as to tumor inoculation, host, and dosage are given in Tables 5 and 6. Only the "optimal" dose of each experiment is used for Tables 5 and 6. If evaluation is by tumor weight, that non-toxic dose is optimal which gives the highest inhibition; a dose is considered toxic if more than one out of 6 animals die during the experiment or if the average loss of body weight due to drug during the experiment (9 days) is more than 10%. If evaluation is by survival time, that dose is optimal which gives the highest percentage of survival time increase (irrespective of toxicity). When the number of experiments indicated in Table 5 or 6 is more than one, the dose and the antitumor activity are averaged. Inoculation of the tumor is on day 0. For evaluation by tumor-weight inhibition, animals were killed on day 9.

The weight of the Walker tumor in control rats was usually between 15 and 25 g on the day of evaluation, weight of sarcoma 37 about 1 g, of sarcoma 180 0·5–1 g. Survival time of untreated mice inoculated with the P-815 and P-1534 leukemias was between 13 and 15 days. SDF control mice, inoculated s.c. with $10^6$ L-1210 cells, had a survival time of 8·96 days (mean value of 34 groups of 6 mice; standard deviation of the average of each group is $\pm 1 \cdot 03$ days, standard error of the mean being $\pm 0 \cdot 18$ days). In DBA/2 mice, survival time was 7·64 days when $10^6$ L-1210 cells were implanted s.c.; the survival time of i.p. inoculated ($10^6$ L-1210 cells) SDF mice was 7·85 days. There was one survivor in the control groups of the L-1210 experiments with VM 26 (among more than 400 mice).

As can be seen from Table 5, sarcomas 37 and 180 are inhibited to a limited degree with a maximum of 72% reduction of tumor weight compared to controls. The Walker tumor shows more response: tumor weights of animals treated with the "optimal" dose are usually less than one-third of those of the controls, and with intravenous application of VM 26 twice weekly (1, 3 and 6 days after tumor implantation) a nearly complete inhibition of tumor growth is obtained. Macroscopic growth of the Ehrlich ascites tumor is prevented completely (at least for 9 days) with intraperitoneal administration of the drug, while survival time of the animals was increased by only 75% and there were no cures (survivors). In leukemias P-815 and P-1534, increase in life span was moderate (30–59%).

In leukemia L-1210, considered as the most reliable animal predicting test system for cytostatic drugs [13], increase in life span due to treatment with VM 26 varies, depending on site of inoculation and treatment schedule (Table 6). From Table 6, the following points emerge: good activity also with delayed treatment (i.e. start of treatment 5 or 7 days after inoculation of the tumor; at this stage, the tumor is palpable); prolongation of survival time of between 54 and 286% when the tumor is implanted into its isologous host, DBA/2 mice; significant prolongation of survival time of mice inoculated intracerebrally with L-1210; indefinite survival of some mice implanted with L-1210 and treated with VM 26, especially if the inoculum is small or if it is given i.p. Mice were assumed to be cured ("survivors") when they survived for at least 4 weeks after the last drug application and showed no signs of tumor growth. Survivors were usually challenged with a second s.c. inoculation of L-1210 cells. Those which received $10^4$ cells mostly survived, while they usually succumbed to a second inoculation of $10^5$ or $10^6$ cells, although their survival time was, after that second tumor implantation, longer than that of simultaneously inoculated control mice; this also is indication of a certain degree of immunity against L-1210 cells. VM 26, on the other hand, also possesses some immunosuppressive properties [14]. There were no survivors among L-1210 inoculated, VM 26-treated DBA/2 mice.

Of some importance from the practical point of view seems the fact that in all animal tumor systems used, VM 26 has a better effect when it is administered not daily but every third day or twice weekly.

To give an impression of the dose–response relationship, the results of two representative examples of experiments with L-1210 are shown in Figs. 6 and 7. The results with the doses 1·6 and 12 mg/kg respectively of these two experiments are included in Table 6.

Per cent kill [15] of L-1210 cells by single application of VM 26 can be calculated from increase in life span under the following conditions: doubling time of L-1210 cells in the respective host is known; treatment does not change the doubling time of surviving cells; animals die when the number of leukemic cells per mouse reaches a certain level. By inoculating SDF mice with different numbers of L-1210 cells and recording survival time we found a doubling time of 0·61 days. We then took the average ILS produced by a single application of the optimal dose of VM 26 in 8 experiments (not all of which are listed in Table 6) where drug was injected at different times (average 3·2 days) after s.c. tumor inoculation. Applying the concept of Skipper [15] we obtain a 99·998 per cent kill (Table 7).

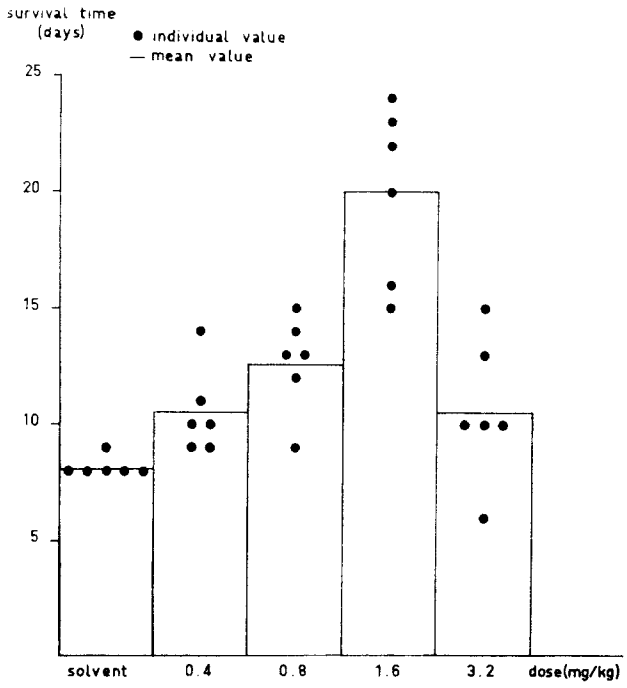


*Fig. 6. Representative example of experiment with daily i.p. VM 26 treatment, beginning 1 day after s.c. inoculation of $10^6$ L-1210 cells into SDF mice. ILS with the optimal dose (1·6 mg/kg per day) is 146%.*

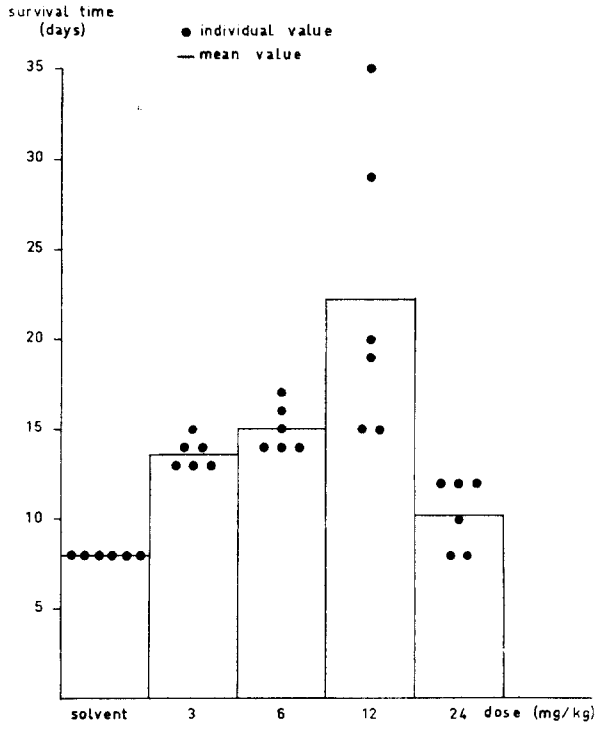


*Fig. 7. Representative example of experiment with twice weekly VM 26 i.v. treatment, beginning 1 day after s.c. inoculation of $10^6$ L-1210 cells into SDF mice. ILS with the optimal dose (12 mg/kg per injection) is 176%.*

*Table 7. Kill percentage of L-1210 cells in SDF mice with single application of VM 26. Doubling time is 0·61 days. The values are averages of 8 experiments*

| | |
|---|---|
| Dose, mg/kg i.p. | 41 |
| LD-17 (S × D mice, i.p.) | 51 |
| Day of treatment | 3·2 |
| Survival of controls, days | 9·05 |
| ILS, per cent | 106 |
| ILS, days | 9·60 |
| ILS, generations | 15·7 |
| Kill, per cent | 99·998 |
| Survivors, one cell out of 53,000 | |

Using the same concept for multiple treatment we obtain, with the optimal dose for daily application (2·6 mg/kg i.p., ILS 121%, see Table 6), a 64% kill with each of the 12 doses; with the optimal dose for application twice weekly (17 mg/kg i.p., ILS 165%, see Table 6) we obtain a 94% kill with each of the six doses.

## DISCUSSION

The activity of VM 26 in inhibiting cell multiplication *in vitro* is of the same order of magnitude as that of the spindle poisons colchicine, podophyllotoxin, and vinblastine, and of some other drugs as well (Table 1). Actinomycin D also has, for inhibition of proliferation of P-815 mastocytoma cells, an ED-50 of 0·005 mg/l. The only drug with a significantly higher activity tested by us in this system is verrucarin A, a fungus product [16]

Microscopical pictures and mitotic counts in fibroblast cultures treated with VM 26 clearly show that this drug, in contrast to podophyllotoxin, podophyllic acid ethyl hydrazide (SPI), colchicine, and vinblastine, prevents cells from entering mitosis. It seems to have preserved its spindle poison activity, since in the first half hour after addition of the drug, there begin to appear c-mitotic metaphases while ana- and telophases disappear. There is, however, practically no accumulation of arrested metaphases since the inhibition of beginning of mitosis by VM 26 prevents this. The spindle poison effect thus becomes visible earlier than the "preprophase activity", probably because the latter affects a process occurring earlier in the cell cycle but becoming recognizable morphologically later only through failure to enter prophase.

If inoculation in fibroblast cultures is continued beyond 3 hr the ED-50 for inhibition of cell division (as calculated from the index of ana- and telophases) decreases. This is also indication of a considerable time interval between addition of VM 26 and visible effect on mitosis; no such decrease occurs with pure spindle poisons. It also seems that spindle poison activity of VM 26 is weaker, i.e. occurs at higher concentrations only, than preprophase activity.

Another argument in favor of a point of attack of VM 26 earlier than mitosis in the cell cycle is the observation that this drug inhibits $H^3$-thymidine uptake by P-815 cells by more than 50% in short-term experiments (2–3 hr) *in vitro* at concentrations of 0·1 mg/l [17]. No such inhibition was found with podophyllum compounds of the pure spindle poison type [18]. The effects of colchicine (and its derivative demecolcine) and vinca alcaloids on nucleic acid synthesis have been reviewed and discussed by Savel [19] and Sartorelli and Creasey [20]. In some of the reported investigations (some of which indicate inhibitions of DNA synthesis) such high concentrations or doses of drug have been used that it is doubtful whether the effects obtained are specific for the antimitotic activity. Since spindle poisons act at a moment of the cell cycle when chromosomes have been duplicated it can be assumed that they have no significant primary effect on DNA synthesis. This is in line with results of exploratory short-term experiments *in vitro* [21] in which no depression of $H^3$-thymidine uptake into proliferating P-815 mastocytoma cells was found with demecolcine treatment (0·1 mg/l) and only slight inhibition with vinblastine at the same concentration. These findings are in agreement with those of Taylor [22] who also used an *in vitro* system with low concentrations ($10^{-7}$ M) of colchicine and short incubation times to exclude non-specific and secondary effects. It thus seems that VM 26 differs from pure spindle poisons also in its primary effect on nucleic acid synthesis.

Tumor inhibition *in vivo* by VM 26 is moderate in the solid mouse tumors tested, sarcomas 37 and 180. Complete macroscopic tumor inhibition is produced when mice with Ehrlich ascites tumors are treated i.p.; but there remain a number of living tumor cells since in an experiment with evaluation by survival time, all animals died (Table 5), and ILS was only 75%. Walker carcinosarcoma, considered by Goldin *et al.* [13] one of the best animal predicting systems for human tumors, shows better response to VM 26 than the solid mouse tumors, a finding valid also for several other agents [23]. According to the results of Table 6, the dosage schedule of treatment twice weekly is superior to daily treatment in leukemia L-1210 of the mouse, but data with larger time intervals between injections are insufficient to prove that

the twice weekly schedule is optimal. According to Table 7 (mean ILS of 9·6 days with single treatment of 41 mg/kg) it could be assumed that injections of 41 mg/kg at intervals <9·6 days would eventually lead to cures. The single experiment performed with larger than 3–4 days intervals between injections, namely one week (see Table 6), does not point in that direction, however. ILS produced by VM 26 is somewhat less when L-1210 cells are implanted s.c. into the isologous host (DBA/2 mice) than in the $F_1$-hybrids, but the results are of the same order; they are not different for the two hosts when the tumor cells are implanted i.p. For mice inoculated intracerebrally with L-1210, ILS is quite significant, but considerably inferior to animals with s.c. or i.p. implanted tumors.

The therapeutic index of VM 26 in L-1210, which is about 7, compares favorably with several clinically used agents listed by Skipper and Schmidt [23]. As to spindle poisons, colchicine, podophyllotoxin, vinblastine, and some of their derivates were found to be ineffective in L-1210 when treatment was delayed to day 7 after tumor implantation [24]. In our hands, podophyllotoxin, podophyllic acid ethyl hydrazide, and demecolcine produced only little (<40%) or no ILS in L-1210 with daily treatment starting on day 1. Survival time increase obtained with vinblastine and vincristine in L-1210 with early treatment is below 60% in hybrid mice and very moderate or zero in DBA/2 mice [25], while it is always above 60% with VM 26, except upon intracerebral inoculation.

The mechanism of action of 4′-demethyl-epipodophyllotoxin thenylidene glucoside is not known at the biochemical or molecular level, but the chemical structure suggests that this drug possibly has points of attack in proliferating cells different from those of other cytostatic substances. The likelihood therefore exists that no cross resistance with known anticancer or antileukemic drugs will be found.

## SUMMARY

*The new podophyllotoxin glucoside derivative VM* 26 (*4′-demethyl-epipodophyllotoxin thenylidene glucoside*) *was found to have a high cytostatic activity in cell cultures. Contrary to previously known podophyllum compounds it does not lead to metaphase accumulation but inhibits entry of cells into mitosis or destroys cells preparing for mitosis. One of its biochemical effects is inhibition of thymidine uptake. Antitumor activity (reduction of tumor weight or increase in life span, ILS) was between* 30 *and* 100% *in several transplantable animal tumors, including Walker carcinosarcoma and leukemia P-*1534. *The effect of VM* 26 *in mouse leukemia L-*1210 *was investigated in more than* 40 *experiments with different treatment schedules. ILS was* 121% *for daily i.p. treatment in s.c. inoculated L-*1210 *and it went up to* 373% (*with* 8 *survivors in* 2 *experiments*) *for treatment twice weekly in i.p. inoculated* L-1210. *ILS produced by VM* 26 *in intracerebrally implanted L-*1210 *was* 36–56%.

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# Factors Determining Cell Killing by Chemotherapeutic Agents *In Vivo*—I.

## Cyclophosphamide

L. M. VAN PUTTEN and P. LELIEVELD

*Radiobiological Institute TNO, Lange Kleiweg* 151, *Rijswijk Z.H., The Netherlands*

THE EFFECTIVENESS of a chemotherapeutic agent is determined by the ratio of the number of tumour cells to that of normal host cells killed by its action. The selective anti-tumour effect of some cytostatic drugs has been explained by the finding that the activity of the drug was limited to cells in DNA synthesis or in mitosis. Such a property makes a drug more effective in killing rapidly growing lymphoma cells than resting haemopoietic stem cells in the mouse [1]. For other drugs which have a similar selective action on rapidly proliferating cells the mechanism of action is as yet unknown. An investigation of cell survival after exposure to such a drug, cyclophosphamide, is the subject of this report

### MATERIAL AND METHODS

*Cell survival*

The criterion for cell killing was the loss of proliferative capacity and the assays used were directed at the capacity of the tumour cell for production of a tumour upon inoculation into a new host, and for the haemopoietic precursor cell for its capacity to form a spleen colony when injected into an irradiated isologous recipient.

The properties of the mouse osteosarcoma C22LR, which originated in a female (C57BL/Rij × CBA/Rij)$F_1$ hybrid mouse in 1957, and has been serially transplanted in similar hybrid mice, have been described earlier [2].

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The present studies were performed in 1969 with passages 76–82 derived from passage 75 cells, which had been stored in liquid nitrogen since 1965. Reinhold's method [3] of preparing cell suspensions from solid tumours was used. The tumour cell survival assay was performed by the endpoint dilution method. Briefly, a single cell suspension was prepared from the tumour by mild trypsinization and agitation, and after enumerating the cells in a haemocytometer, suitable dilutions were made and serially increasing numbers of cells were injected subcutaneously into isologous recipients. The mice were palpated for the occurrence of tumours at the inoculation sites at 7–14 day intervals at least up to day 100, or until no new tumours were found to appear in a 30-day period. From the number of tumours registered in the recipient it was possible to determine the TD50, that is the number of cells that must be inoculated in order to produce 50% tumours at the injection sites. From a comparison of the results in pools of treated and control tumours a surviving fraction may be calculated [4].

The spleen colony technique of Till and McCulloch [5] was used with minor modifications. The bone marrow of treated donors was flushed from the femurs with cold Tyrode's solution. If necessary 20–50 units of heparin and 500–1000 dornase units of DNAse were added to the suspension to prevent coagulation or clumping.

After filtration through nylon gauze, suitable dilutions of the suspensions were prepared and

injected in 1/2 ml volume into the tail vein of (C57BL/Rij × CBA/Rij)$F_1$ hybrid males that had been irradiated with 750 rads 1–2 hr earlier. Nine days later the recipients were killed and the spleens were fixed in Telleyesniczky's fluid. Under a dissecting microscope the colonies with diameters of 0·25 mm or more were counted.

In each experiment spleens were also studied from mice that had been irradiated but not injected with bone marrow cells. In 162 such spleens only 2 colonies were found. The number of colony-forming units (CFU) per normal femur was 6750±238 (S.E.) in 35 assays.

The fraction of surviving cells was calculated as the ratio of the mean number of CFU recovered per femur of treated to that of untreated control mice.

Rapidly proliferating spleen-colony forming cells were also exposed to chemotherapeutic agents. For this purpose suspensions were made from the spleens of mice that had been lethally irradiated (750 rad) and injected with a standard amount of bone marrow cell suspension 7 days earlier. Just as the femurs of such mice — as used by Bruce *et al.* [6, 7] for a similar purpose — the spleens of these animals contain exponentially increasing numbers of CFU (doubling time 22 hr) ranging on day 7 from 8000 to 12,000 per spleen. Drugs were administered to groups of 5 such mice at each dose level and the survival of rapidly proliferating CFU was calculated as the ratio of the mean number of CFU per donor spleen of treated to that of simultaneously assayed control mice. The rapid proliferation of the CFU in the spleens of these mice was confirmed by their increased sensitivity to vinblastine and tritiated thymidine compared to normal bone marrow CFU.*

*Radiation*

The irradiation of tumours *in vivo*, of femur-bone marrow cell donors and of spleen-colony assay recipients was performed as whole body irradiation at a dose rate of 55 rad/min delivered by a General Electric Maxitron 250 kVp X-ray machine, operated under the following conditions: 30 mA, 250 kVp, HVL of the beam 2·3 mm Cu. The dose was measured in the center of tissue equivalent mouse phantoms and all radiation was administered under simultaneous monitoring by a reference dosimeter mounted on the radiation cage. Assay for cell survival was performed immediately after irradiation, except when stated differently.

*Unpublished observations.

*Chemotherapy*

Cyclophosphamide† and vinblastine were administered intraperitoneally to mice. When single doses were administered, these were calculated on the basis of the body weight of individual mice (this varied from 20 to 30 g). In the fractionated-dose studies the mean body weight was used to determine the dose which was given to all mice. Unless otherwise indicated bone marrow and tumour-cell donors were sacrificed 16–20 hr after the administration of chemotherapeutic agents and if fractionated doses were given, 16 hr after the last dose.

## RESULTS

Comparative studies of the sensitivity of tumour cells and CFU to different agents are presented in Fig. 1. It is evident that haemopoietic stem cells are much more sensitive than tumour cells to whole body irradiation. Vinblastine and cyclophosphamide are much more effective in killing tumour cells than bone marrow stem cells. When these results are compared with similar data of Bruce *et al.* [1] for a mouse lymphoma, two differences are noted. In the first place it is evident that the osteosarcoma is less radiosensitive than the lymphoma; this is not only due to a difference in intrinsic cell sensitivity but also to the fact that the solid osteosarcoma contains a fraction of anoxic cells which are less sensitive to radiation than well-oxygenated cells [2], whereas the lymphoma, which grows diffusely infiltrating in the host femur, seems to contain almost no anoxic cells. The second point of interest is the seemingly biphasic curve for the survival of haemopoietic cells after cyclophosphamide. A more detailed analysis of these data is presented in Fig. 2, line A, which shows that the data are best fitted by a straight line for the dose range between 50 and 400 mg/kg. The best fitting line deviates significantly from the origin. This implies that between 0 and 100 mg/kg there must be an early steep portion in the dose–effect curve. Such a steep curve at low doses might be the consequence of the presence of a subpopulation among the colony forming cells, which is much more sensitive to the drug. For a number of other agents, subpopulations with a higher sensitivity have been found to consist of cells in a sensitive phase of the cell cycle [1] and for comparison a study of the sensitivity of rapidly proliferating CFU to the drug seemed indicated. The

† ® Endoxan was kindly made available by the Asta Werke through Multipharma, Amsterdam.

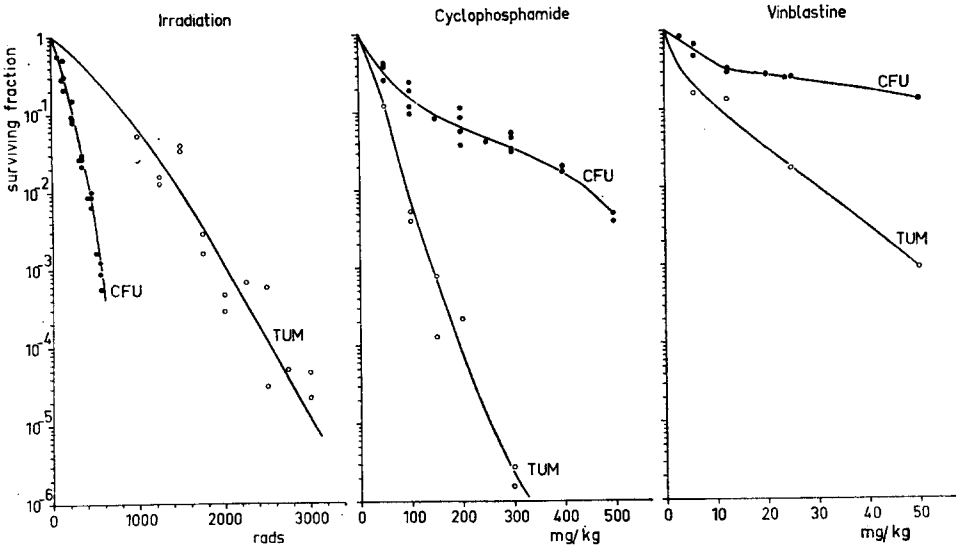


*Fig.* 1. *Survival curve for osteosarcoma cells (TUM) and bone marrow stem cells (CFU) after* in vivo *treatment with total body irradiation or intraperitoneal administration of a single dose of chemotherapeutic agents.*

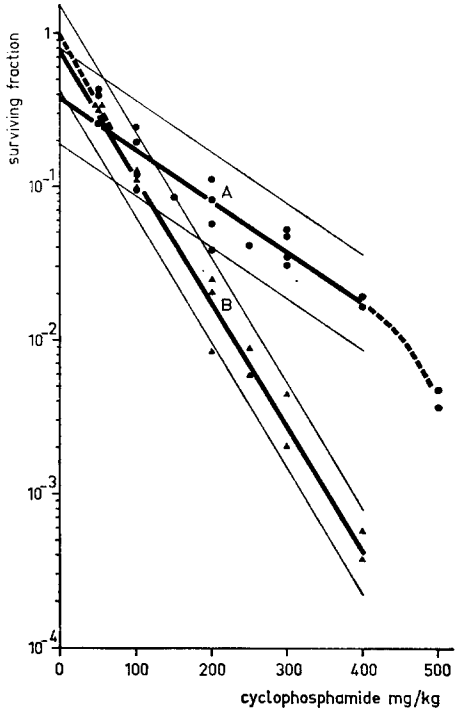


*Fig.* 2. *Detailed survival data for spleen colony forming cells after injection of cyclophosphamide. Line A and* ● *refer to the survival of normal bone marrow CFU. Line B and* ▲ *represent the survival after cyclophosphamide treatment of rapidly proliferating colony forming cells in the spleens of mice that had been irradiated and injected with isogenic bone marrow cells 7 days earlier. The lines have been calculated by the method of least squares and the parallel lines enclose the* 95% *confidence intervals.*

results are presented in line B of Fig. 2, and they show that these rapidly proliferating CFU are clearly more sensitive to cyclophosphamide.

A number of possible mechanisms for this finding are conceivable. It might be explained as a consequence of an increased sensitivity of cells when exposed in a certain phase of the cell cycle. The rapid proliferation might cause all cells to reach the sensitive phase in the time period during which an effective drug level was maintained. However, Mellet [8] has shown that the elimination of radioactive labelled cyclophosphamide and its activation products in the mouse is very rapid; by 3 hr after administration of 200 mg/kg body weight the activity in the blood is a small fraction of the peak level. This rapid excretion makes it unlikely that all cells could pass through a

sensitive phase of the cycle in the short period for which high drug levels are present.

More information on this point might be obtained if drug levels are maintained for longer periods of time and for this purpose four doses of cyclophosphamide were given at 2½ hr intervals to normal and to irradiated mice, bone marrow-treated mice having rapidly proliferating haemopoietic stem cells in the spleen. The survival curves are presented in Fig. 3. It is evident that for the normal recipients there is no increased effectiveness of fractionated doses of cyclophosphamide; on the contrary, perhaps there is a slightly higher

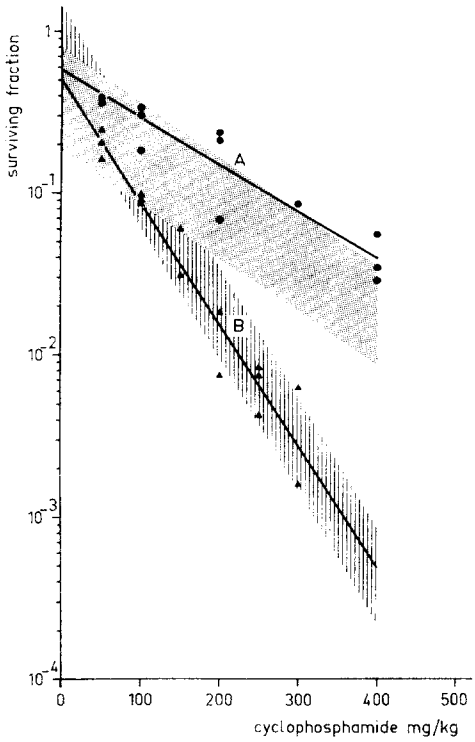


*Fig. 3. Survival of haemopoietic stem cells after fractionated treatment with cyclophosphamide. Each donor received 4 injections at 2½ hr intervals. The total dose is plotted against survival. Line A and ● represent the data for survival of CFU of normal bone marrow; line B and ▲ the data for repopulating spleen. The shaded areas refer to the confidence limits of Fig. 2 for comparison.*

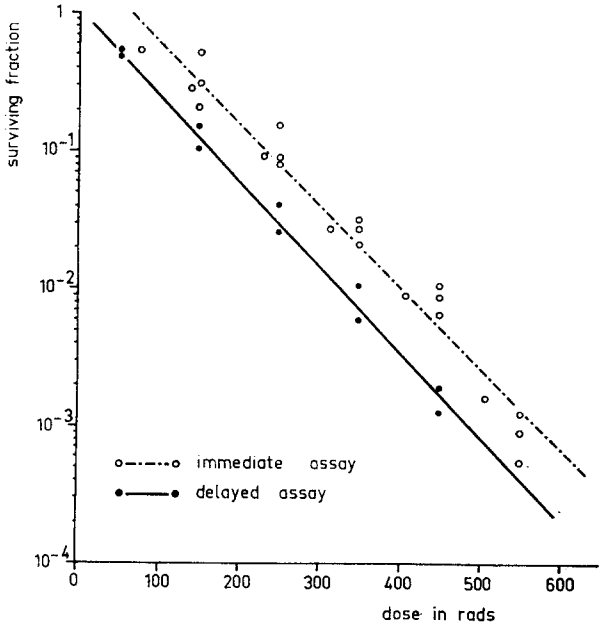


*Fig. 4. Dose–effect curve for the survival of bone marrow stem cells after irradiation* in vivo. *The assay for the number of surviving colony forming cells per femur was made either immediately after irradiation or 24 hr later. The delay causes a further loss of surviving colony-forming cells.*

surviving fraction than after equal total doses administered in a single injection.

The results of fractionated exposure of rapidly proliferating cells are very similar to those of single doses. Since there is certainly no higher sensitivity under these conditions, there is no support for the hypothesis that cells are more sensitive during a limited period of the mitotic cycle. An alternative explanation for the biphasic curve was suggested by the possibility of loss of CFU before assay, as was found after X-irradiation of donor mice. Data on the effect of 24 hr delay in CFU assay in the femur of irradiated recipients have been reported earlier [9]. The results are summarized in Fig. 4. These studies show a parallel downward displacement of the survival curve as a consequence of the delay in CFU assay. In studies comparing the kinetics of bone marrow CFU after irradiation, similar decreases in CFU recovery have been described by various authors [10–12], and on the basis of parallel studies on a decrease of the number of CFU recoverable from the spleens of lethally irradiated mice after injection of bone marrow cells [13] the conclusion has been drawn that this decrease is not a consequence of delayed CFU killing by radiation, but rather of a transformation of the colony-forming cells to differentiated daughter cells which have lost the capacity to form visible colonies.

Whatever the mechanism may be, it is clear that in the time interval between irradiation and late assay there is a dose-independent loss of CFU. A similar loss of CFU during the interval between the cyclophosphamide action and the CFU assay may have been responsible for a parallel downward displacement of the true drug dose–survival curve and may thus have caused its biphasic form. This possibility was confirmed in another series of studies in which the cyclophosphamide treated mice were sacrificed for CFU assay 3 hr after drug injection. The results are presented in Fig. 5. They show that the survival curve is indeed displaced upward, compared to line A of Fig. 2. The early steep part of the curve is absent, suggesting that the mechanism causing the biphasic curve when survival is assayed after 16–20 hr may be similar to that operating in irradiated donors. Apparently, the dose–effect curve for the CFU survival if assayed early, is a simple exponential. Variation of cell sensitivity over the mitotic cycle is apparently not responsible for the biphasic curve of Fig. 2. It should be noted that another deviation from linearity

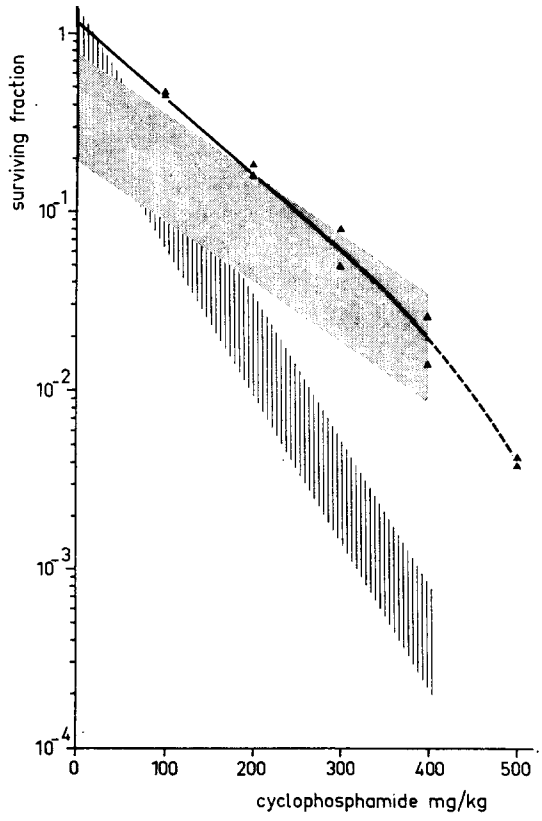


*Fig. 5. Dose–effect curve for normal bone marrow colony forming cells when the assay for survival is made 3 hr after injection of cyclophosphamide.*

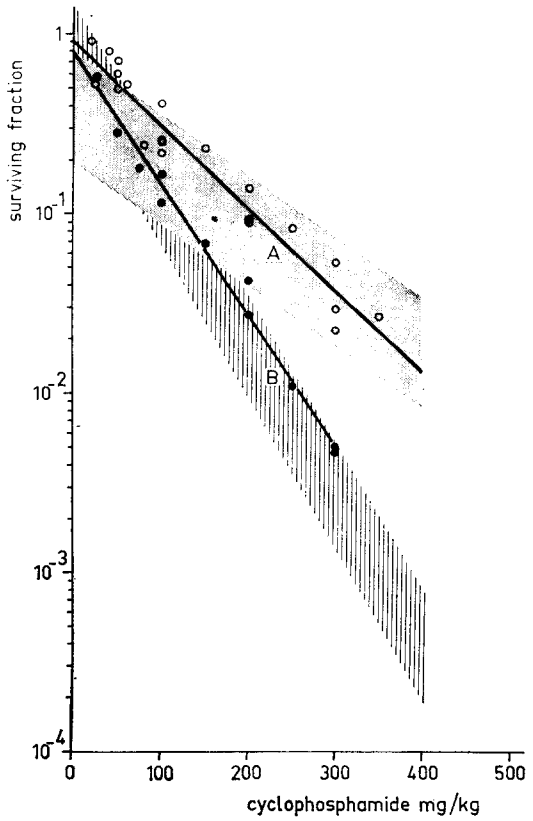


*Fig. 6. Lines A (○) and B (●) are the dose–effect curves for normal bone marrow colony-forming cells and rapidly proliferating colony-forming cells from repopulating spleens respectively after exposure to cyclophosphamide in the assay recipients.*

has appeared in Fig. 5: the downward curvature, which was also noted in line A of Fig. 2 for the 500 mg/kg dose, seems in this experiment to start already at 400 mg/kg.

It might be argued that the observed differences in sensitivity between CFU populations in normal mouse bone marrow and in rapidly repopulating spleens might be a consequence of different degrees of transformation of the inactive cyclophosphamide into active metabolites in the two different types of host. To investigate this possibility another study was made using an assay for the sensitivity of different cell populations to the drug when exposed in the assay recipients. For this purpose irradiated mice were injected, both with cell suspensions intravenously and with cyclophosphamide intraperitoneally. The injections were given within a few seconds of each other and this was always done within 30 min after irradiation of the recipients. Nine days later the recipients were sacrificed for spleen-colony counts as usual. The survival curves are presented in Fig. 6, lines A and B. It is clear that the difference in slopes of sensitivity between the two types of CFU is maintained also when we have no reason to assume differences in cyclophosphamide transformation.

The parameters of the best fitting lines (calculated by the method of least squares) to the various experimental results are presented in Table 1. It is evident that the slopes of the survival curves, obtained for normal bone marrow CFU are similar, although there is a tendency to steeper slopes if the time interval between drug administration and assay is shortened. The parameters obtained from the survival data of rapidly proliferating CFU belong to a different group in which the slopes are steeper independent of the method of assay. The sensitivity of the tumour cells is again more than a factor of 2 higher than that of the rapidly proliferating spleen-colony forming cells.

## DISCUSSION

The outstanding work of the Toronto group [1, 6, 7] on the survival kinetics of haemopoietic cells after exposure to chemotherapeutic agents has presented us with a new tool for the study of the mechanisms of action of these drugs. In the present studies this technique has been applied to the analysis of the detailed cell survival kinetics of haemopoietic stem cells after cyclophosphamide. So far two components of the curve have been

*Table 1. Parameters of survival curves for haemopoietic colony-forming cells and osteosarcoma cells after cyclophosphamide treatment*

| Colony-forming units from normal bone marrow | $T$* (hr) | $D_o$† (mg/kg) | $n$‡ |
|---|---|---|---|
| Assay 16–20 hr after one single injection § | 18 | 126 | 0·4 |
| Assay 16 hr after last of 4 doses at 2½ hr interval | 21 | 150 | 0·6** |
| Assay 3 hr after one single injection¶ | 3 | 102 | 1·2** |
| Drug administered to assay recipients | 0 | 94 | 0·9** |
| Rapidly proliferating colony-forming units from chimaera spleen | | | |
| Assay 16–20 hr after one single injection | 18 | 54 | 0·8** |
| Assay 16 hr after last of 4 doses at 2½ hr intervals | 21 | 58 | 0·5** |
| Drug administered to assay recipients | 0 | 60 | 0·8** |
| Osteosarcoma cells | | | |
| 20 hr after one single injection | | 23 | 0·3** |

*$T$ = mean time interval between drug administration and CFU assay.
†$D_o$ = is the reciprocal of the slope of the survival curve. It represents the dose increment which reduces survival to 37%.
‡$n$ = extrapolation number to zero dose of the best fitting exponential.
§Data for 500 mg/kg excluded (see text).
¶Data for 400 and 500 mg/kg excluded (see text).
**Not significantly different from 1·0 ($P > 0·05$).

distinguished: in the first place an essentially exponential survival curve which differs in slope for rapidly proliferating and for resting cells, and in the second place an artificially lowered extrapolation number, which has been shown to be associated with the delayed sampling for surviving cells. A third component has only briefly been mentioned; in the dose–effect curves for resting stem cells from normal mouse bone marrow it was noted that after administration of 400 or 500 mg/kg of the drug the cell survival was lower than expected on the basis of exponential extrapolation. The effect was more pronounced and occurred at lower drug levels if the interval between administration and assays was shorter. Since this shorter interval implies that also the interval between drug administration and the transition to rapid proliferation will be shorter, this finding suggests the possibility that either a fraction of the drug, or a fraction of the latent damage caused by large quantities of the drug might still be present in the cells at the time when the cells enter the mitotic cycle. The rapid excretion of cyclophosphamide and its metabolites [8] makes the former possibility unlikely, but latent damage has indeed been described [14] after treatment with alkylating agents.

There is evidence that the lethal action of some of the bifunctional alkylating agents is associated with alkylation of DNA and that in micro-organisms this lethal action is prevented when DNA synthesis is blocked by a lack of essential amino acids or uracil [14]. Repair of lesions caused by the alkylating agents has been demonstrated in mammalian cells [15] and it has been shown that micro-organisms resistant to difunctional alkylating agents show rapid repair but no increase in initial alkylation [16]. If the action of metabolites of cyclophosphamide is based on analogous processes, the following mechanism of action might explain the greater activity versus rapidly proliferating cells. Alkylation by the agents leads to latent damage of DNA. This is turned into lethal damage if the cells enter the phase of DNA synthesis, but a slow repair mechanism may prevent this if there is a long time interval between alkylation and the next DNA synthesis period. The minor differences in the slopes of the survival curves for normal bone marrow stem cells, depending on the time interval between contact with the drug and the proliferation stimulus associated with the assay procedure are in agreement with these suppositions. Since the rapidly proliferating cells are already in cell cycle, a similar relationship does not apply to these populations.

Apparently not all bifunctional alkylating agents show this difference in effect towards resting and rapidly proliferating cells. Nitrogen mustard gave a similar slope of survival for normal haemopoietic cells and lymphoma cells, although the extrapolation number may be different [1]. A comparison of other alkylating agents is in progress in order to learn whether the property of differential action based on speed of proliferation is associated with better clinical effectiveness.

Finally, it should be noted that the transplantable osteosarcoma is far more sensitive to

cyclophosphamide than the rapidly proliferating haemopoietic cells (see Table 1). Since the rapidly proliferating colony forming cells are capable of producing a spleen colony of about $10^6$ cells in 9 days, their cycle time is probably not longer than 12 hr. It is not likely that the tumour cells proliferate much faster and other factors than the speed of proliferation must be involved in the higher effectiveness of good chemotherapeutic agents against malignant cells. It is hoped that the comparison of normal and malignant cells at similar proliferation rates will permit a better insight in the mechanisms involved.

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## SUMMARY

*The effect of various chemotherapeutic agents* in vivo *on the survival of mouse haemopoietic spleen colony-forming cells and osteosarcoma cells is compared and a detailed study is made of the dose–effect curve of cyclophosphamide for the haemopoietic stem cells in normal-mouse bone marrow and in rapidly repopulating spleen. Different parts of the dose–effect curve could be attributed to different mechanisms and it was evident that cyclophosphamide is more effective against rapidly proliferating cells. After elimination of an artefact caused by the assay technique it could be shown that the greater activity versus rapidly proliferating cells is not caused by a higher sensitivity when the cells are exposed in a particular phase of the mitotic cycle, as is the case for instance with vinblastine. The hypothesis is advanced that DNA alkylation by cyclophosphamide or its metabolites may take place in any part of the cell cycle, but that cell killing will occur only if the lesion has not been repaired before DNA synthesis takes place. Finally the results show that only part of the difference in activity of cyclophosphamide versus normal bone marrow stem cells and tumour cells may be ascribed to differences in the rate of cell proliferation; other discriminating mechanisms must be involved.*

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# Inhibition of Polyoma-Virus Oncogenesis in Rats by Polyriboinosinic-Ribocytidylic Acid*

M. VANDEPUTTE, S. K. DATTA, A. BILLIAU† and P. DE SOMER
*Rega Institute, University of Leuven, 3000 Leuven, Belgium*

## INTRODUCTION

POLYRIBOINOSINIC-ribocytidylic acid (In.Cn), a synthetic double-stranded RNA, is a very efficient inducer of interferon in tissue cultures as well as in the whole animal [1]. The induction of interferon in the host cells by In.Cn may explain the resistance against lytic viral infections in animals and in tissue culture [1–3].

Furthermore, In.Cn was also shown to be active against different oncogenic viruses. Its effect against certain of these, like the murine sarcoma [4] and murine leukaemia viruses [5], suggest an interferon-mediated mechanism. However, experiments with adenovirus 12 in hamsters [6] indicate that other mechanisms like immunologic stimulation rather than interferon induction may be responsible for the protective effect observed. Furthermore, In.Cn has also been shown to reduce the growth rate of transplantable tumors and even to cause regression of some tumors [7]. As most of these tumors were either "virus-free" or non-viral induced tumors, the action of In.Cn is hard to explain solely on the basis of its interferon-inducing capacity.

In the experiments to be reported, we verified the effect of In.Cn on polyoma oncogenesis in rats. In previous studies it was demonstrated that the induction of polyoma tumors is strongly dependent on the immunologic competence of the host and that small changes in this competence lead to a marked effect on the tumor incidence [8, 9]. Furthermore, there is little, if any, replication of infective virus in the rat and the polyoma virus persists only for a short period of time in the host tissues [8]. For these reasons, we have chosen this tumor-virus system in order to dissociate, by a properly timed administration of In.Cn, the interferon-mediated effect of the drug from other possible mechanisms of action like stimulation of the immunologic reactivity or a direct antitumor effect.

## MATERIAL AND METHODS

*Animals*

Rats of the inbred Wistar R strain were used.

*Viruses*

Polyoma virus was grown on mouse embryo-fibroblast cultures (MEF) and prepared by the method of Crawford [10]. The virus was titrated with the plaque method of Dulbecco and Freeman on MEF cells [11].

*Polyriboinosinic-ribocytidylic acid* (In.Cn)

Single-stranded homopolymers, polyriboinosinic acid (In) and polyribocytidylic acid (Cn), were purchased from P-L Biochemicals Inc., Milwaukee, Wisconsin. They were dissolved in Dulbecco's phosphate buffered saline at a concentration of 1 mg/ml. Double-stranded In.Cn was obtained by combining equal amounts of homopolymer solutions. They were heated at 90°C for 30 min and then slowly cooled to room temperature. Hypochromicity

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measured at 245 mμ was more than 40%. Biological activity of these solutions was tested by a viral interference test on primary rabbit kidney cells. A yield reduction assay using vesicular stomatitis virus (VSV) as a challenge was used [12]. Neomycin sulphate was included in the medium to increase antiviral activity [13]. The lowest active concentration of the In.Cn preparations in this test was 0·0055 μg/ml.

In order to ascertain that In.Cn induces interferon in rats, female Wistar R rats (150 g) were given 25 or 125 μg of In.Cn intraperitoneally. Blood samples were taken 3 hr later, and serum interferon was determined by a VSV-yield reduction assay on secondary rat embryo cells [12]. Circulating interferon amounted to 2200 and 3000 units respectively.

*Autopsy*

At the end of the experiment all surviving rats were killed after bleeding at the retro-orbital plexus for the determination of leucocytosis and of the titer of hemagglutination antibodies [8].

Rats developing clinical signs of tumor growth before the end of the experiment were processed in the same way. The tumors as well as the lymphoid organs were fixed in Bouin's fluid and further processed for histological examination.

## RESULTS

*Effect of In.Cn inoculated subcutaneously on the induction of tumors by polyoma virus in rats*

In a first experiment, rats of the R inbred strain were injected intraperitoneally (i.p.) 48 hr after birth with $5 \times 10^6$ PFU polyoma virus. One group (gr. 1) of rats was treated subcutaneously (s.c.) with 50 μg In.Cn. one day before the virus inoculation and with progressively increasing doses (from 50 to 100 μg) afterwards, taking into account the increase in body weight. Three injections of In.Cn were given per week during two weeks (the total amount of In.Cn inoculated s.c. was 430 μg spread over 6 injections).

In a second group (gr. 2), the In.Cn treatment was started one week after polyoma virus inoculation. The first dose consisted of 80 μg In.Cn, the subsequent doses being increased progressively to 180 μg. Three injections were given per week during two weeks (the total amount of In.Cn inoculated s.c. in group 2 was 670 μg, spread over 6 injections).

Group 3 (control group) was treated s.c. with phosphate buffered saline (PBS) following the same schedule as for group 1. The control group (gr. 3) was spread over the littermates from both experimental groups (gr. 1 and 2). All surviving animals were bled and autopsied 6 to 7 weeks after virus inoculation.

As shown in Table 1, a significant ($P<0·01$) reduction in tumor incidence was recorded amongst the rats in which the In.Cn treatment was started one week after virus inoculation (gr. 2) compared to the control group. No significant decrease ($0·05<P<0·1$) in the total tumor incidence was observed in the group 1, treated with In.Cn before and after polyoma virus inoculation. However, in the group 1 the incidence of cerebral hemangiomas was remarkably low (4 out of 26 autopsied) compared to the control group (24 out of 32 autopsied). Furthermore, whereas the incidence in kidney sarcomas was not significantly different between group 1 and group 3 (controls), the size of the kidney tumors found in the In.Cn-treated group was much smaller than in the controls. The mean diameter of the kidney sarcomas in group 1 was 14·4 mm (range 7·5–18 mm) against a mean diameter of 20·2 mm (range 14–24 mm) in the control group (gr. 3).

No significant difference was observed in the titer of hemagglutinating-inhibiting antibodies (HAI) between the rats tested in the different groups. In this experiment the leucocytosis was not tested. The body-weight curve of the rats treated with In.Cn was found to be identical

*Table 1. Effect of In.Cn inoculated s.c. on the polyoma-tumor-incidence in R rats*

| | Treatment | Number inoculated | Number autopsied | Number and % with tumors | Tumor type: cerebral hemang. | kidney sarcoma | osteo-sarcoma |
|---|---|---|---|---|---|---|---|
| Group 1 | In.Cn before and after virus inoculation | 37 | 36 | 26 (72) | 4 | 26 | 0 |
| Group 2 | In.Cn started 7 days after virus inoculation | 35 | 34 | 21 (61·7) | 14 | 21 | 0 |
| Group 3 | Controls (PBS-treated) | 34 | 32 | 29 (90·6) | 24 | 28 | 3 |

to the body weight curve recorded in the control group.

*Effect of In.Cn injected i.p. at different times before or after polyoma-virus inoculation*

In the first experiment we observed a marked protective effect on the polyoma-tumor-incidence of In.Cn treatment started one week after virus inoculation, but no significant decrease in the total tumor incidence in rats treated before and after virus injection. In this last group, only a decrease in the number of cerebral hemangiomas and in the size of the kidney tumors was recorded.

Therefore, we repeated this experiment using a higher dose of In.Cn and also included a group whereby the In.Cn treatment was postponed for as long as 3 weeks after virus inoculation.

Rats of the R inbred strain were inoculated i.p. 3 days after birth with $10^6$ PFU-polyoma virus. One group (gr. 1) of rats was treated i.p. with 100 μg In.Cn 24 hr before the virus inoculation and with progressively increasing doses (from 100 to 200 μg) afterwards. The dosage was increased proportional to the increase in body weight of the rats. Three injections of In.Cn were given per week at two days interval during two weeks (a total amount of 960 μg In.Cn, given in 6 injections, was inoculated i.p.).

In a second group (gr. 2), the In.Cn treatment was started one week after the virus inoculation. The first dose consisted of 160 μg and was progressively increased to 300 μg. Three i.p. injections of In.Cn were given per week during two weeks (a total amount of 1·36 mg of the In.Cn was given in 6 injections).

In a third group (gr. 3), the In.Cn treatment was started as late as 21 days after the virus inoculation. The first dose in this group consisted of 400 μg In.Cn and was progressively increased to 800 μg at the last (6th) injection. Like in the other experimental groups, the rats received 3 i.p. inoculations of In.Cn per week, during two weeks. The total amount of In.Cn given to group 3 was 3·45 mg, given in 6 injections.

The last group (gr. 4) in this experiment was the control group. These rats received PBS instead of In.Cn, following the same schedule as for group 1. The control rats were spread over the litters from the different experimental groups. All surviving animals were bled and autopsied 6–7 weeks after virus inoculation.

As shown in Table 2, a significant ($P<0·01$) reduction in tumor incidence was recorded in the experimental groups treated at different times with In.Cn compared to the control group (gr. 4). Even when the In.Cn treatment was postponed for as long as 21 days after virus inoculation, the compound proved to have a remarkable protective effect on the polyoma-virus-tumor induction in rats. As at that time polyoma virus is no longer demonstrable in significant amounts in the animals as will be discussed later, one must consider the possibility that In.Cn has a direct anti-polyoma-tumor effect.

*Effect of In.Cn on transplanted syngeneic polyoma-tumor cells in R rats*

In this experiment the possibility of a direct antitumor effect by In.Cn was verified on transplantable polyoma-tumor cells. Twenty female R rats of 80 g were inoculated s.c. with $6\times10^6$ syngeneic polyoma-tumor cells in the right flank. These tumor cells derived from a kidney sarcoma induced in a R rat by polyoma virus and cultured *in vitro*. The tumor cells were free of polyoma virus as no virus could be recovered from the tissue culture and the rats inoculated with these cells never develop H.A.I. antibodies in their serum.

When the 20 R rats inoculated with these polyoma-tumor cells had developed small subcutaneous nodules at the site of injection,

*Table 2. Effect of In.Cn inoculated i.p. on the polyoma-tumor-incidence in R rats*

| | Treatment | Number inoculated | Number autopsied | Number and % with tumors | Tumor type | | |
|---|---|---|---|---|---|---|---|
| | | | | | cerebral hemang. | kidney sarcoma | osteo-sarcoma |
| Group 1 | In.Cn before and after virus inoculation | 28 | 28 | 1 (3·6) | 0 | 1 | 0 |
| Group 2 | In.Cn started 7 days after virus inoculation | 30 | 30 | 1 (3·3) | 0 | 1 | 0 |
| Group 3 | In.Cn started 21 days after virus inoculation | 29 | 28 | 8 (28·6) | 4 | 5 | 0 |
| Group 4 | Controls (PBS-treated) | 64 | 60 | 54 (90) | 25 | 54 | 19 |

they were divided in two groups of 10. The first group (gr. 1) was inoculated three times a week, s.c., in the left flank with 400 μg In.Cn. Five out of ten In.Cn-treated rats were autopsied two weeks after the first inoculation of In.Cn (these 4 rats received 6 inoculations of 400 μg of In.Cn). The remaining 5 rats were treated for one more week (receiving a total of 9 inoculations of 400 μg of In.Cn) before being autopsied. The control group (gr. 2) received PBS instead of In.Cn. In this group half of the rats were also killed 14 days after the first injection of PBS and the remaining 5 rats were autopsied one week later. Before autopsy, the body weight curve of all animals was recorded and blood was taken to detect the presence of H.A.I. antibodies. The size and wet weight of the tumors were verified as well as the macroscopic aspect of the tumor (necrosis, hemorrhage). Two fragments of each tumor were fixed in Bouin's fluid for histological examination: one fragment was taken at the periphery and one out of the central portion of the tumor. No significant difference in tumor size or tumor weight was recorded between the two groups. The mean tumor weight in group 1 was 1·31 g (range 0·85 to 1·38 g) for the rats killed after 2 weeks of In.Cn treatment and 1·35 g (range 0·78 to 1·96 g) for the control rats (gr. 2).

In the rats autopsied one week later the mean tumor weight was 10·56 g (range 7·1–14·6 g) for the In.Cn treated rats (gr. 1) and 10·4 g (range 7·3–14·3 g) for the control rats (gr. 2). Neither was there any significant difference in the body-weight curve or in the macroscopic or microscopic aspect of the tumors examined in both groups.

All rats had negative H.A.I. antibody titers, indicating the absence of infectious polyoma virus in the transplantable tumor cells.

## DISCUSSION

In.Cn treatment, started either before or 7–21 days after virus inoculation, prevented the induction of tumors by polyoma virus in rats.

In.Cn was shown to induce the interferon mechanism in cultured rat cells [13] as well as after intraperitoneal injection in the rat (cf. Material and Methods). Therefore, the inhibitory effect of In.Cn on polyoma virus-induced tumors in rats, when polymer treatment is started before virus inoculation, may be explained on the basis of interferon induction. This finding is also in accordance with previous studies on the protective effect of In.Cn on other oncogenic viruses, like murine sarcoma [4] and murine leukaemia viruses [5]. However, the observation that In.Cn treatment started seven or twenty-one days after polyoma-virus inoculation, is still very effective, is hard to explain solely on the basis of an interferon antiviral mechanism. Previous studies [8] have shown, indeed, that polyoma virus does not multiply to significant levels in rat tissues and that infectious polyoma virus is no longer demonstrable in the rat 2–3 weeks after virus inoculation. Although one has to consider the possibility that interferon may in certain cases exert a direct antitumor effect [14], our results strongly suggest that other mechanisms than interferon induction are responsible for the protective effect of In.Cn on polyoma-tumor induction.

Amongst the different mechanisms of action to be considered, the induction of an increased immunologic response in the host or a direct antitumor effect seem the most likely. The absence in our experiments of any demonstrable effect of In.Cn on an established and active growing syngeneic transplantable polyoma tumor makes the possibility of a direct antitumor effect less plausible. Yet it does not exclude the possibility that In.Cn treatment started before or immediately after the inoculation of the transplantable polyoma-tumor cells may exert an inhibitory effect on the tumor growth. This experiment is now in progress.

Previous studies [8, 9] have stressed the important role of the immunologic response and more particularly of the cellular immunity in the defence mechanisms against polyoma-tumor induction in rats. As other experiments clearly indicate that In.Cn enhances the immunologic response [15–17], it seems plausible that such an increase might also be responsible for the protection obtained in the polyoma system in rats when the treatment by this drug is postponed for as long as 3 weeks after virus inoculation. A similar immunologic mechanism has been proposed by Larson *et al.* [6] to explain the protective effect of In.Cn on tumor induction in hamsters by adenovirus type 12. However, in their experiments In.Cn treatment proved to be active only when started before virus inoculation and had no inhibitory action when given afterwards. Conceivably, the adenovirus 12-induced tumors in hamsters are less responsive to immunologic factors than our polyoma system in rats.

## SUMMARY

*Polyriboinosinic-ribocytidylic acid (In.Cn) markedly reduced the incidence of tumors in rats injected with polyoma virus. This protective effect was observed not only when In.Cn was given before and immediately after the virus inoculation but was also recorded when In.Cn treatment was started 7–21 days afterwards. This finding strongly indicates that other mechanisms than an interferon antiviral mechanism are responsible for the observed protective effect. As In.Cn did not seem to exert a direct antitumor effect on transplantable polyoma tumors in the rat, it is very likely that its inhibitory action on polyoma oncogenesis in rats is due to an increase of the immunologic response in the host.*

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# Cell-Free Transmission of Murine Myeloid Leukaemia

T. TANAKA* and A. W. CRAIG

*Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester 20, England*

## INTRODUCTION

SINCE Gross [1] reported the transmission of murine leukaemia by cell-free filtrate, several types of virus-induced leukaemia have been recognized in mice, such as lymphocytic leukaemia [2], erythroleukaemia [3, 4] and myeloid (chloro-) leukaemia [5]. In addition, Gross [6] and Kaplan and his colleagues [7] produced evidence that the radiation-induction of lymphocytic leukaemia or lymphoma was associated with the viral aspects. Transmission of myeloid leukaemia by cell inoculation [8] and its induction by radiation [9] have been reported. On the other hand, there has been little investigation of viral induction of myeloid leukaemia [10, 11]. This paper reports the methods used to obtain an enhancement of the induction of myeloid leukaemia by cell-free filtrates.

## PROCEDURES AND RESULTS

*Leukaemia line*

The RFM/Un mice bearing radiation-induced myeloid leukaemia were transferred to this laboratory from the Oak Ridge National Laboratory, Oak Ridge, Tennessee, in January 1966. The leukaemic cell line has been passaged at 7–10-day intervals by intravenous injections of $10^5$ to $10^6$ leukaemic spleen cells in suspensions.

*Induction of leukaemia using supernatant*

Deep-frozen (at −70°C) leukaemic spleen and liver were rapidly thawed, minced together, ground with washed sand, suspended in Hanks' solution (20–30% w/v) and spun twice at 1500 **g** and 2500–3000 **g** for 20 min at 4°C. The resulting supernatant was injected intraveneously into 1–3·5 months old RFM/Un mice, 0·2–0·3 ml per mouse.

The overall incidence of myeloid leukaemia after injection of supernatant was 38·4% with an average latency of 36·8 days. Forty-one of 102 males (40·2 %) and 45 of 122 females (36·9%) developed leukaemia, with an average latency of 36·5 and 37·0 days, respectively. There was no significant difference in the leukaemic incidence related to the age or sex (Table 1a).

*Induction of leukaemia using filtrate*

Supernatant was filtered through Millipore filters (25 mm diameter) of 0·45 or 0·8μ porosity. The resulting filtrate was injected intravenously into 123, 3–5·5 months old, RFM/Un mice, 0·4–0·5 ml per mouse. These were all negative (Table 1b). Due to difficulties in the filtration of supernatant directly through small porosity of filters (e.g. 0·45 or 0·8μ porosity), a 1·2μ filter was employed for a pre-filtration to eliminate coarse particles in the supernatant. Subsequently, this filtrate was filtered using a 0·8μ filter; the resulting filtrate was injected into 193, one month old mice, without any success. A 0·8μ filter was thoroughly washed with Hanks' solution after filtering the supernatant which had been pre-filtered. Two out of 6 mice injected with this solution developed myeloid leukaemia with a latency of 25 and 43 days (Table 1c).

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**Present address:* Department of Experimental Pathology and Cancer Research, School of Medicine, University of Leeds, Leeds LS2 9NL, England.

*Table* 1. *The incidence of myeloid leukaemia after injection of supernatant or cell-free filtrate prepared from* RFM/Un *leukaemic organs*

| | No. of mice injected | Sex | Age at injection (months) | Medium used for organ extracts | Millipore filter pore size (μ) | No. of leukaemia induced | Induction time (days) |
|---|---|---|---|---|---|---|---|
| Supernatant | | | | | | | |
| a. | 102 | M | 1–3·5 | Hanks | | 41 | 36·5 (16–56) |
| | 122 | F | 1–3·5 | Hanks | | 45 | 37·0 (16–68) |
| Filtrate | | | | | | | |
| b. | 13 | M, F | 3·5–4 | Hanks | 0·45 | 0 | |
| | 110 | M | 3–5·5 | Hanks | 0·8 | 0 | |
| c. | 193 | M | 1 | Hanks | 1·2–0·8* | 0 | |
| | 6 | M | 1 | Hanks | Solution prepared from washed Millipore filter (0·8 μ) | 2 | 25, 43 |
| d. | 323 | M | 1–1·5 | Hanks | 3·0, 1·2, 0·8, 0·45† | 0 | |
| | 18 | M | 1–1·5 | Saline | 1·2, 0·8 | 0 | |
| | 8 | M | 1–1·5 | Saline | 3·0 | 4 | 18, 19, 20, 20 |
| e. | 62 | M, F | 1–3 | Calf serum | 3·0, 1·2 | 0 | |
| | 19 | M, F | 1–3 | Horse serum | 3·0, 1·2 | 0 | |
| | 14 | M, F | 1–3 | Dist. water | 1·2 | 0 | |
| | 29 | M, F | 1–3 | Dist. water | 3·0 | 1 | 22 |
| | 28 | M, F | 1–3 | Saline | 1·2 | 0 | |
| | 13 | M | 1–3 | Saline | 3·0 | 2 | 17, 25 |
| | 15 | F | 1–3 | Saline | 3·0 | 2 | 18, 28 |
| f. | 9 | F | 1 | Saline | 3·0 | 9 | 15 (4 mice) 16 (3 mice) 20 (2 mice) |

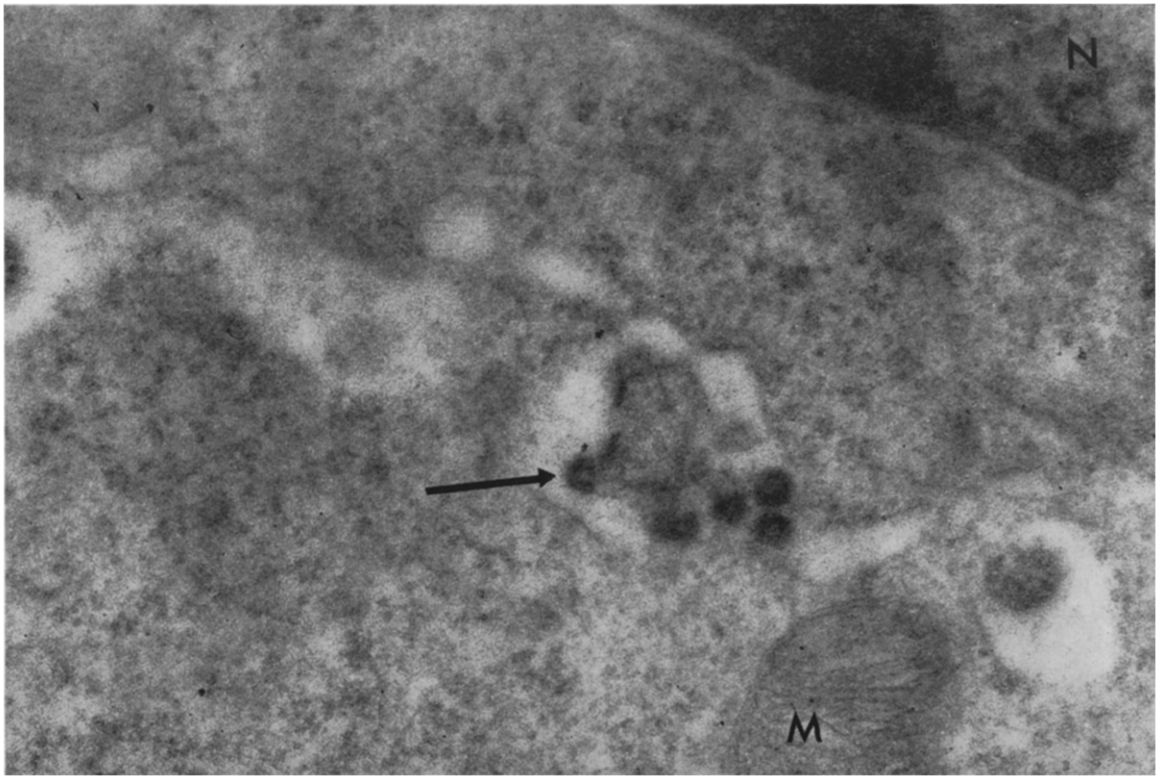
*A 1·2μ filter was used for a pre-filtration: subsequently this filtrate was filtered using a 0·8μ filter.

†A 3·0μ filter was used for a pre-filtration: subsequently this filtrate was filtered using either 1·2, 0·8 or 0·45μ filter.

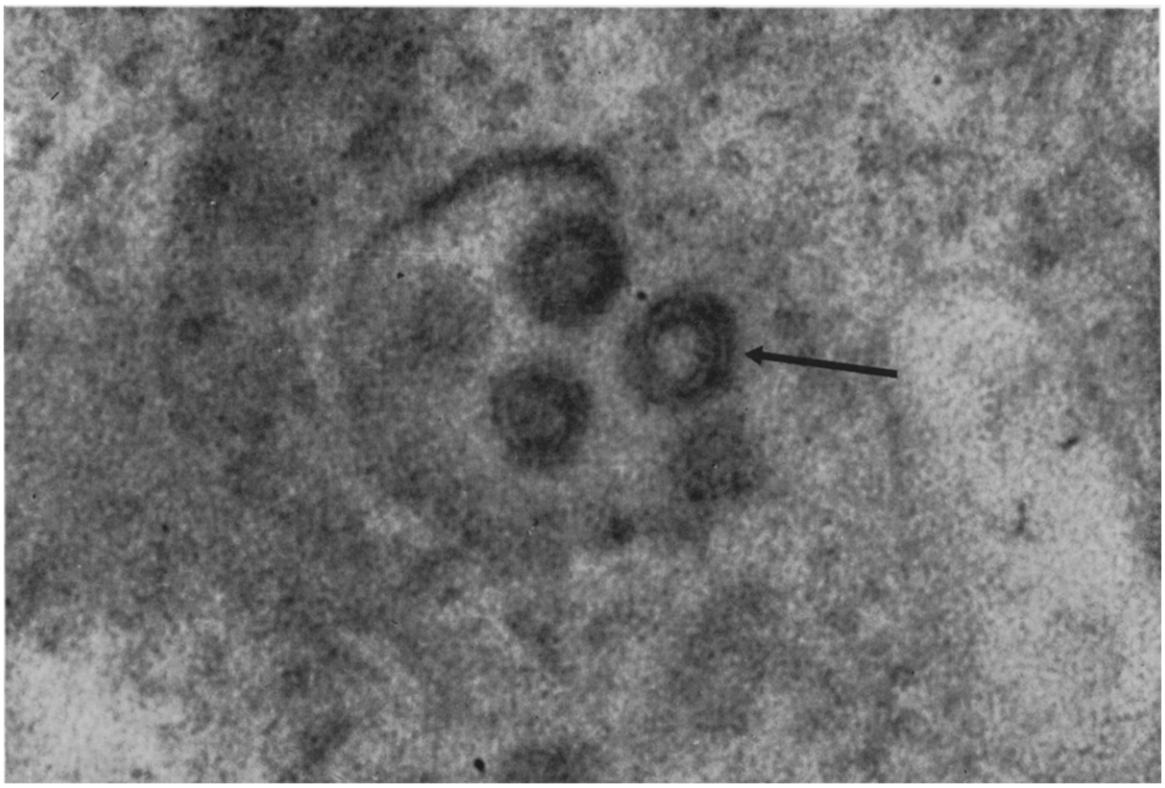
In view of the apparent influence of filter porosities in the yield of leukaemia, the filtrate obtained initially, using a 1·2μ filter, was divided into 3 fractions. The first fraction was used directly, without any further filtration. The second fraction was filtered again, using a 0·8μ filter, and the third fraction using a 0·4μ filter. In the subsequent experiments, the use of different porosities was extended further and a 3·0μ filter was used for pre-filtration; filters were tested and found to be impervious to *E. coli*. A total of 323, 1–1·5 month old mice were injected with the filtrates described above. None of these treated mice developed leukaemia. Up to this time, Hanks' balanced salt solution had been exclusively used for suspending minced and ground leukaemic organs. On a particular occasion, however, 4 out of 8 mice injected with 3·0μ-filtrate suspended in physiological saline developed myeloid leukaemia between 18 and 20 days (Table 1d).

In view of the additional influence of the medium used for the preparation of cell-free filtrates on the yield of leukaemia, an attempt was made to test various media for this possibility. Minced and ground organs were suspended in either diluted calf or horse serum, distilled water or physiological saline. A total of 180, 1–3 months old mice were injected with these filtrates. One of 29 mice injected with distilled water-treated filtrate (3·0μ filter) and 2 of 13 males and 2 of 15 females injected with saline-treated filtrate (3·0μ filter) developed leukaemia between 17 and 28 days (Table 1e).

The liver, spleen, mesenteric lymph node, lung, heart (containing coagulated blood), kidneys and ovaries from a leukaemic mouse, induced by injection of aqueous filtrate, were processed together, as previously described, suspended in saline, filtered through a 3·0μ filter and injected into 9, one month old mice. All the mice developed myeloid leukaemia




(*c*). *Two particles with tail-like structure* (*arrows*) (× 240,000).



(*d*). *Double particles sharing a common core* (*arrow*). (× 120,000).

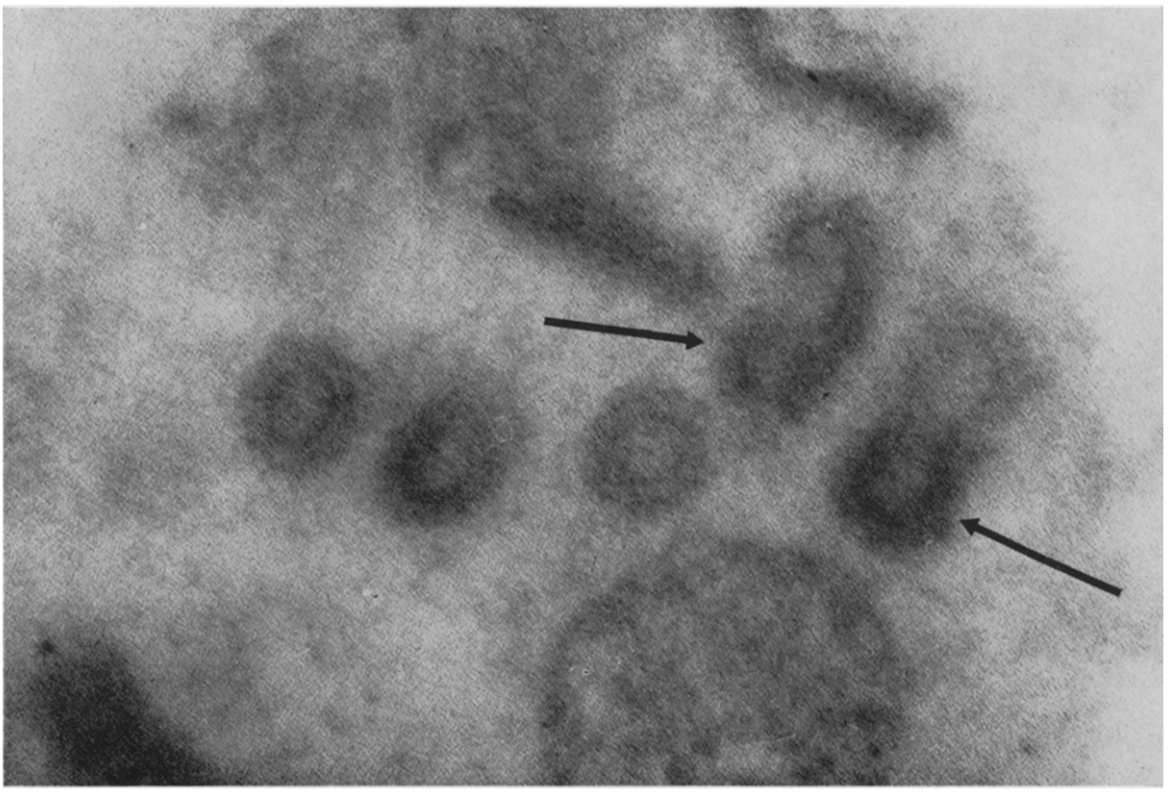
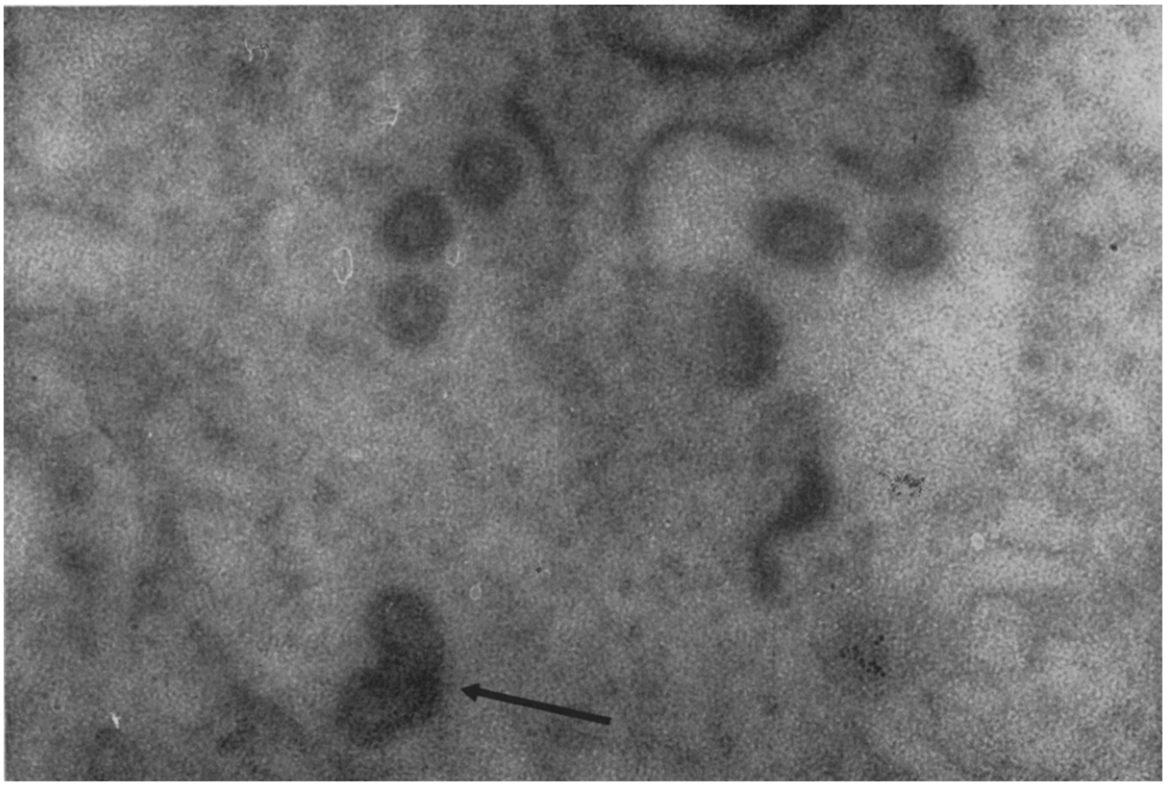


*Fig.* 1. *Virus particles present in the spleen of myeloid leukaemia mice* (RFM/Un).

(*a*). *A particle budding from a vacuolar membrane* (*arrow*) (×60,000). *Nucleus* (N) *and mitochondrion* (M).



(*b*). *A particle having three membranes* (*arrow*). (×180,000).

(to face p. 330)

with a latency of 15–20 days (Table 1f). Small pieces of tissue from the leukaemic spleen and liver in this experiment were processed for electron microscopic studies. Virus particles were observed in cytoplasmic vacuoles (Fig. 1). These were mainly doughnut type (Type C in Bernhard classification), having an electron-lucent centre surrounded by inner and outer membranes with an average diameter of 50 and 100mμ respectively. Sometimes, particles appeared to bud from a vacuolar membrane. Six normal animals (1–3 months old) were examined at the same time and no virus particles were found. Further studies using these leukaemic organs are now in progress.

## DISCUSSION

The overall incidence of myeloid leukaemia following injections of supernatant was 38%. However, the possibility of transplanting intact leukaemic cells still exists if centrifuged extracts are used as the inoculum. Furth and Kahn [12] and Skipper *et al.* [13] showed that a single viable cell can induce leukaemia in mice. In the present experiments, however, care was taken to store leukaemic organs at −70°C for at least 2–3 days in order to attempt to destroy all viable cells before use. According to Furth [8], the use of myeloid leukaemic cells frozen at −35°C for 30 min reduced the leukaemic incidence to zero.

When the supernatant was filtered, injecting this filtrate dropped leukaemia incidence to virtually nil. This is apparently similar to the experience of Jenkins and Upton [11]. According to their data, the incidence of myeloid leukaemia after injection of supernatant into unirradiated, 2–2·5 months old mice was 20% with a latency of 12–164 days. The material used was derived from leukaemic mice of 6th to 23rd transplant generations. By using filtrate prepared with a Selas 03 filter, however, the incidence decreased to 4% with a latency of 9–130 days. These results clearly indicate that a considerable number of virus particles is likely to be held on the filter surface or inside the filter pores, probably depending on quality and porosity of the filters as well as the medium used for organ extracts. This view is supported also by the present report, in which the highest leukaemia incidence was achieved using a 3·0μ porosity Millipore filter and physiological saline solution for the medium.

Originally, Gross [14] prepared filtrates using Berkefeld or Selas filter candles and Graffi [15] used a G4-Schott glass filter. The pores of these porcelain or glass filters are not of the same size, nor do they hold to the same dimensions throughout their complete course. The membrane filter, such as Millipore, was considered to be more uniform in pore size and would adsorb less virus particles than the above filters. Nevertheless, according to Friedman *et al.* [16], and England [17], the pores in the Millipore vary greatly in diameter and are tortuous channels. Cliver [18] compared the efficiency of filters using enteroviruses and concluded that losses of virus particles in filtration were due to adsorption to the filter membrane and were governed by its chemical composition. Cellulose nitrate membrane, such as Millipore filter, in the absence of interfering substances (e.g. serum), adsorb enterovirus at a pore size 285 times the virus diameter. On the other hand, cellulose triacetate filters, made by the Gelman Instrument Company, adsorb virtually no enterovirus when the pore size exceeds the virus diameter by a factor of more than 3. Some support for this view can be found in the data of Jenkins and Upton [11]. One of 5 mice injected with filtrate prepared using a membrane filter (Gelman) with a porosity of 2 μ developed myeloid leukaemia, whereas only 7 of 178 mice injected with filtrate from a Selas filter developed the disease.

Cliver [18, 19] was able to minimise adsorption of enterovirus to membrane matrix by the incorporation of newborn bovine or chicken serum into the virus suspension at the time of filtration, or by pretreating the membrane with serum. It must be pointed out, however, that some animal sera — such as calf and horse — apparently contain an antibody-like substance against viruses, e.g. enterovirus [20]. Wallis and Melnick [21] reported enhancement of adsorption of enterovirus particles (28 mμ in average diameter) on a 0·45 μ Millipore filter by increasing the salt concentration (mainly NaCl and $MgCl_2$) in the culture medium. The filter porosity was, in this case, more than 15 times larger than the particle diameter.

With respect to the filters used in this study, a high incidence of leukaemia was obtained when a 3·0μ filter was used. The size of major avian or murine leukaemia virus particles is approximately 100 mμ [22]. This agrees well with the size of myeloid leukaemia virus reported by Parsons *et al.* [10] and in this study. The pore size required in this report is, therefore, unquestionably large; for example, Friend leukaemia virus, with a diameter of 87 mμ [23], can be filtered with a 0·22μ porosity of membrane filter without any reduced titration [24].

In view of the above complexities, which still remain to be elucidated, the myeloid leukaemia

virus may possess very unique features, probably quite distinctive from either Gross, Moloney, Friend, Rauscher, Graffi or any other known murine leukaemia viruses. Once a high yield of myeloid leukaemia can be obtained constantly in cell-free preparations, the nature of the virus and of the transplantation antigens related to myeloid leukaemia could be clarified by immunological studies. An immunotherapeutic approach would also be feasible, as attempted by Boranić [25] on RFM/Un myeloid leukaemia. At the same time, consideration of the factors for the enhancement of virus yields may be important in the search for possible human oncogenic viruses.

## SUMMARY

*A successful enhancement of the induction of murine myeloid leukaemia by cell-free extracts prepared from leukaemic organs is reported. The overall incidence of myeloid leukaemia by injection of supernatant was* 38%, *with an average latency of* 37 *days. When the supernatant was filtered, injecting this filtrate reduced leukaemia incidence to virtually nil. A series of investigations showed a filtrate prepared using* 3·0 μ *membrane filter and physiological saline to be a suitable way of enhancing virus yields. On a particular occasion, all* 9 *mice thus treated developed myeloid leukaemia with a latency of* 15–20 *days. The results clearly indicate that variable numbers of virus particles were held on the filter surface or inside the filter pores, depending on the quality and porosity of the filters as well as the medium used for organ extracts.*

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# Nucleic Acids-Hybridization Studies in Normal and Cancer Cells*

Y. VALLADARES,* Y. ALVAREZ,† E. TABARÉS and T. PINTADO

*Department of Biology and Biochemistry of Cancer. Instituto Nacional de Oncología. Ciudad Universitaria, Madrid-3, Spain*

## INTRODUCTION

EMPLOYING the agar column technique of Bolton and McCarthy [1] we realized a series of experiments related to nucleic acids complementarity in order to investigate some aspects of the molecular biology of carcinogenesis. It is a well known fact that the degree of complementarity between single-stranded DNA–DNA and DNA–RNA systems may be used to study genetic similarity, philogenic relationship, presence of viral genome in cell genome, and origin and function of cell and virus RNAs through the existence of DNA carrying information for mRNA, sRNA, and rRNA. We shall present here some data based on hybridization procedures employing nucleic acids from normal and cancer cells.

## MATERIAL AND METHODS

*Cells*

DNA was isolated from AV3/INO, TC-CV/INO, TC-SV40/INO and ER-SV40/INO cell lines, as well as from normal human amnion of fresh placentas. AV3/INO cell derives from a clone of a human amnion line [2]; TC-CV/INO established line proceeds from a hamster tumor produced by inoculation of CV virus to newborn animals; TC-SV40/INO line was obtained from a hamster tumor due to SV40 virus inoculation to newborn hamsters; ER-SV40/INO resulted from *in vitro* transformation after inoculation of mouse-embryo primary tissue cultures with SV40 virus preadapted to LLC-$MK_2$ deriv. cell line, consisting of *Macaca mulatta* monkey kidney. The new cell lines are described in another work [3]. Established lines were cultured as monolayers with McCoy R5a growth medium supplemented with 0·5% lactalbumin hydrolyzate, 10% $ARh^+$ human serum and 100 μg of neomycin sulphate per ml of the final solution. Serial subcultures were prepared by seeding $1{\cdot}5 \times 10^5$ cells per ml of growth medium, changing for fresh medium on the fourth day, and subculturing with a 1 to 8 split on the seventh day after detachment with 0·2% trypsin solution.

*Virus.* We used cancer virus, that will be here referred to as CV virus, which is a 120 mμ DNA virus with 60 capsomers (by the method of visible capsomers × 2) developed by us in 1958 starting on virus-free cancer cell DNA from several sources adapted to *in vitro* tissue cultures [4], and vacuolating agent of Hilleman or SV40 virus. The strain of CV virus used proceeds from DNA of a human lung cancer developed in HeLa cell cultures and then adapted to mouse-embryo primary cultures, and is now in its 76th passage in mouse cells and its 86th passage *in vitro*.

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†Scientific collaborator of the "Consejo Superior de Investigaciones Científicas" (Highest Council for Scientific Research) on duty commission at the National Institute of Oncology.
‡Predoctoral research fellow of the Department of Biology and Biochemistry of Cancer.

*Cell DNA*

Cells were collected for each experiment from six Roux flasks having monolayers of 200 $cm^2$ and 70 ml growth medium. The cultures, in logarithmic phase, were detached with 0·2% trypsin solution, centrifuged at 120 g for 5 min in a model HN International centrifuge, the supernatant decanted, the cellular sediment washed with Hanks' balanced salt solution, and centrifuged again at 120 g. The obtained packed cell volume was about 2 ml. The supernatant was decanted and the sediment used for DNA extraction adapting Kirby's procedure [5]. The sediment was resuspended in 6% sodium p-aminosalycilate (Na-PAS), treated with 90% phenol, centrifuged at 24,000 g and 4°C, the aqueous phase removed, DNA precipitated with 2-ethoxiethanol, dissolved again with Na-PAS, reprecipitated with 2-ethoxiethanol, three times dissolved in 4% sodium acetate followed by precipitation with 2-ethoxiethanol, dissolved again to add 30 μg ribonuclease and allowed to stand overnight. Ribonuclease was extracted with phenol, the product centrifuged at 1980 g for 20 min, the aqueous phase collected, the DNA precipitated with 2-ethoxiethanol, and purified from polysaccharides by methoxiethanol extraction from a solution prepared with 2 vol. sodium acetate, and 1·32 vol. of a mixture prepared with 20 parts of M/1 $K_2HPO_4$ and one part of 33% $H_3PO_4$. The product was dialyzed, extracted with a chloroform-octanol (9:1) mixture, centrifuged, the aqueous phase made to 4% sodium acetate, and the DNA precipitated with 2-ethoxiethanol and dissolved in 0·14 M NaCl solution. Aliquots were taken to measure DNA content by UV spectrophotometry at 260 mμ.

*Cell RNAs*

Starting from cultured cells in the same conditions as for DNA obtention, the following procedure was used to obtain the different ribonucleic-acid fractions. The technique was generally realized using 2 ml of packed cell volume. The cells were suspended with an 0·05M NaCl, 0·001M EDTA solution, treated with 90% phenol, stirred in a magnetic stirrer for 60 min, and centrifuged at 27,500 g. RNAs were collected with the upper aqueous phase, and sodium acetate added to make a 2% final solution. Chilled absolute ethanol was used to precipitate rRNA and sRNA, leaving mRNA in the supernatant, that was purified by repeated ethanol precipitation. The precipitated material was dissolved with NaCl-$PO_4$-EDTA solution, potassium acetate added to make a 2% solution, $MgCl_2$ added to get a 0·015 M solution to precipitate rRNA, that was collected by centrifuging at 10,000 g. As for sRNA, it was precipitated from the supernatant by ethanol addition and collected by centrifugation. The obtained rRNA and sRNA were dissolved in NaCl-$PO_4$-EDTA solution. Aliquots from mRNA, rRNA and sRNA fractions were taken and quantitatively determined by UV spectrophotometry at 260 mμ. A scheme of the procedure is given in Fig. 1A.

*Labelling of nucleic acids*

Normal mouse embryos, CV virus-induced mouse cancers, ER-SV40/INO cell line derived from *in vitro* malignant transformation of mouse-embryo cells by SV40 virus, and CV virus, were employed as a source of nucleic acids.

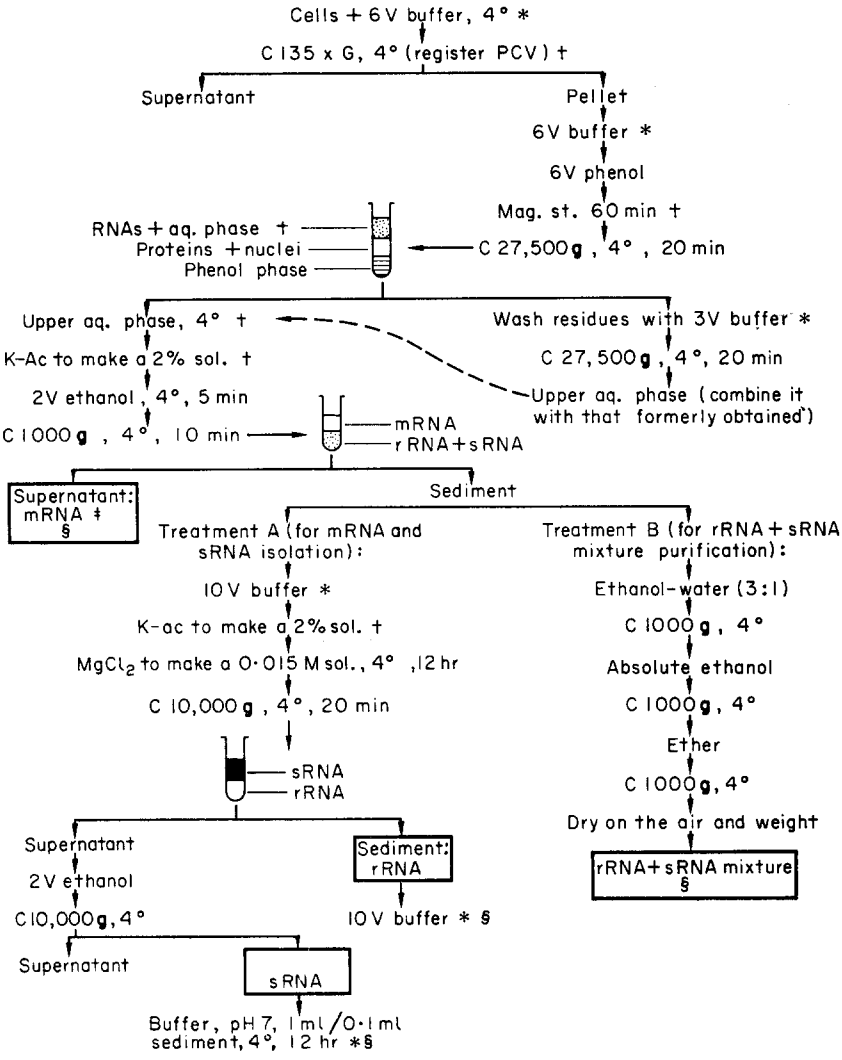
Four-day old cultures in logarithmic growth phase received 0·5 μc methyltritiated-thymidine (TdR-H3) with s.a. 2·0 c/mmol (New England Nuclear Corporation) or 0·5 μc uridine-5-$H^3$ (UR-$H^3$) with s.a. 4·4 c/mmol. Phosphorus-32 labelling was realized by adding 0·5 μc of $P^{32}$ solution (Spanish Nuclear Energy Board) with 100 μc/ml 45 min before nucleic acids isolation. Cells were washed with Hanks' balanced salt solution and collected for DNA or RNA extraction on the 10th day of the culture.

For mRNA labelling *in vivo*, pregnant and tumor-bearing mice were injected intraperitoneally with 0·25 ml of $P^{32}$ solution having a specific activity of $1·65 \times 10^6$ cpm/0·1 ml. After seven hours the embryos were removed from the pregnant animals, and the tumors excised from the tumor-bearing animals, washed with a balanced salt solution (Hanks or GKN), fragmented, washed again, homogenized with a Sorvall omni-mixer homogenizer at 4°C, and then the mRNA extracted as usual. The obtained radioactivity was of 28,357 cpm/μg of mouse embryo mRNA-$P^{32}$, and of 33,402 cpm/μg of tumor tissue mRNA-$P^{32}$.

Twelve-day old mouse embryos were minced, treated with 0·2% trypsin solution to disperse the cells, filtered through gauze, washed with Hanks' balanced salt solution, and seeded at a concentration of 400,000 viable cells per ml. Twelve Roux flasks (200 $cm^2$ culture surface each) received 70 ml each of cell suspension in McCoy R5a growth medium supplemented with 10% calf serum and 0·5% lactalbumin hydrolyazte. Cultures were incubated at 37°C. On the fourth culture day the old medium was decanted, the cells inoculated with 0·7 ml of CV virus suspension from previous cultures

having a TCID50 of $10^{9.5}$ with the nutrient agar plaquing method, fresh growth medium containing 1 μc thymidine-$H^3$ added, and both cell monolayer and growth medium collected twelve days after inoculation. Cells were sedimented by centrifugation, suspended in a little amount of growth medium and broken with ultrasonic waves in a MSE 100 W ultrasonic disintegrator. Cell debris were eliminated by centrifugation, the cell supernatant combined with the growth medium from infected cultures, centrifuged at 200,000 g and 2°C for 2 hr in a model B-60 International Ultracentrifuge, and the pellets collected. The combined virus pellets obtained from 12 Roux flasks gave a packed virus volume of 0·2 ml, that was the usual amount extracted for each experiment.

Viral DNA-TdR·$H^3$ was extracted from the virus pellet following a method based on Kirby's procedure [5]. The pellet was suspended in 6% Na-PAS, stirred with 90% phenol in a magnetic stirrer, centrifuged at 24,000 g and 4°C, DNA precipitated from the aqueous phase with 2-ethoxiethanol, redissolved in 6% Na-PAS, reprecipitated with ethoxiethanol, dissolved with 4% sodium acetate, precipitated again with 2-ethoxiethanol, treated overnight with 500 μg RNase per ml, dialyzed for 24 hr, precipitated with 2-ethoxiethanol and dissolved in distilled water.




* 50 mM NaCl + 1 mM $NaH_2PO_4$ + 1 mM 4Na-EDTA solution

† Abbreviations employed: C, centrifuge; PCV, packed cell volume; Mag. st., magnetic stirrer; aq., aqueous; sol., solution; K-Ac, potassium-acetate

‡ Purified by repeated precipitation of rRNA and sRNA residues with ethanol

§ Aliquots for orcinol reaction and UV spectrophotometry at 260 mμ

*Fig. 1a.*

An aliquot was taken for ultraviolet spectrophotometry and O.D. measured in a Beckman DU spectrophotometer at a wavelength of 2600Å, registering 1945 μg of CV virus DNA-TdR·$H^3$ per ml.

A second aliquot was used for radioactivity calculations by liquid scintillation spectrometry. The registered amount of radioactivity was 11,200 to 16,800 cpm/μg DNA. Depending on the particular experiment, results are referred to absolute counts per minute of radioactivity from virus DNA-TdR·$H^3$, and to DNA amounts in micrograms (μg), nanograms (ng) or picograms (pg).

*Nucleic acids mating*

The general procedure followed was adapted from that of Bolton and McCarthy [1] (Fig. 1b). Five to 10 ml distilled water containing 200 to 800 mg agar (Oxoid agar N° 3) was heated to 100°C. When agar was melted, 5–10 ml distilled water at 100°C and containing 100 to 2500 μg DNA were added and kept at this temperature for 10 min. The agar–DNA was quickly put in an ice bath and once solidified was passed through a stainless steel mesh having 35 holes per square inch. A duplicate sample of this preparation received 3–6 ml of 5 M $NaClO_4$ at 60°C, the DNA collected and its amount estimated by ultraviolet spectrophotometry at 260 mμ. Another two duplicate samples of agar-DNA were washed with 0·3 M NaCl and 0·03 M 3Na-citrate at 60°C and allowed to cool. Five tenths of a milliliter of a solution containing 0·1 to 20 μg of labelled cell DNA or RNA in 0·3 M NaCl and 0·03 M 3Na-citrate buffer were employed. DNA samples were labelled with methyl-tritiated thymidine (DNA-TdR·$H^3$), and RNA with tritiated uridine (RNA-UR·$H^3$) or phosphorus-32 (RNA-$P^{32}$), ultrasonicated to fragment DNA, and heated at 100°C for 10 min to separate DNA strands. The samples were rapidly cooled at 4°C to maintain the strands separated, and then incubated at 60°C for 14 hr. Two of the samples were prepared for immediate column fractionation, and the other two were previously kept overnight in 0·3 M NaCl at 60°C. The samples were put into a chromatography column, 50 ml of 0·3 M NaCl and 0·03 M 3Na-citrate buffer passed at 60°C at a rate of 1 ml per min, and then 5 ml fractions were collected using a model 3400-B-RadiRac LKB automatic fraction collector with 5 ml siphon. Then 50 ml of 1·5 mM NaCl and 0·15 mM 3Na-citrate buffer at 72°C were passed, and again ten 5 ml fractions collected. A little amount of carrier thymonucleic acid was added to each tube, then 5 ml of 30% trichloroacetic acid (15% final solution) at 4°C to precipitate nucleic acids, centrifuged at 1100 g for 15 min in a model RC-2Sorvall refrigerated centrifuge, and the sediment dissolved in 0·2 ml of dis-

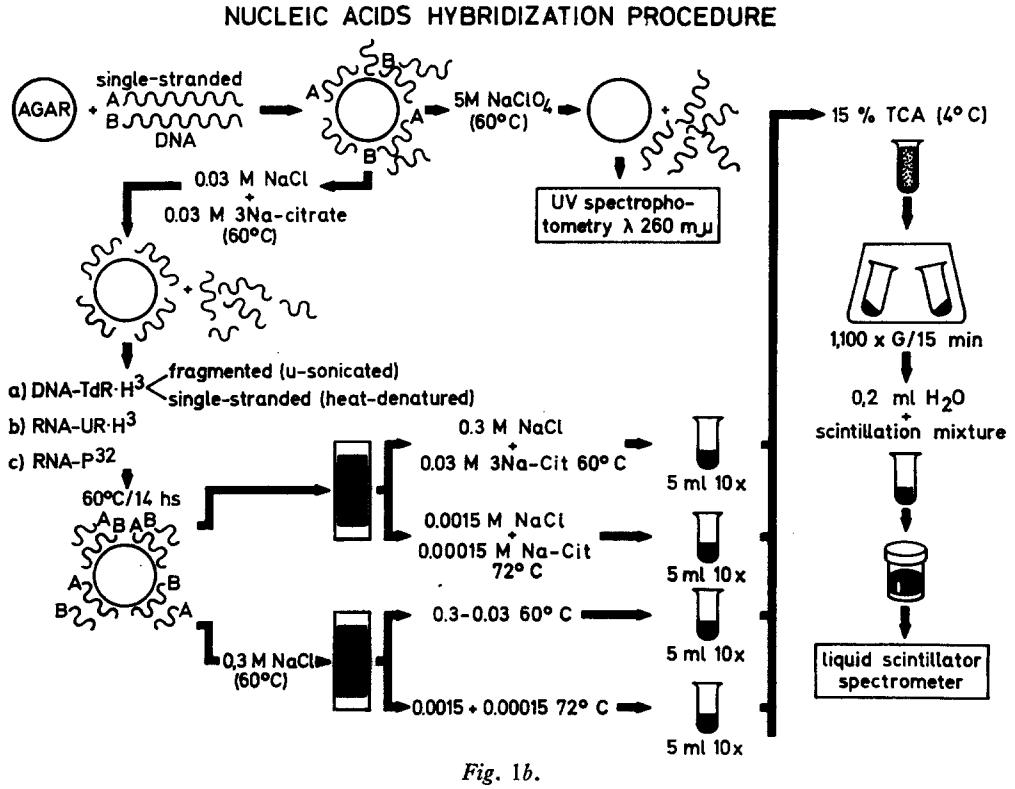


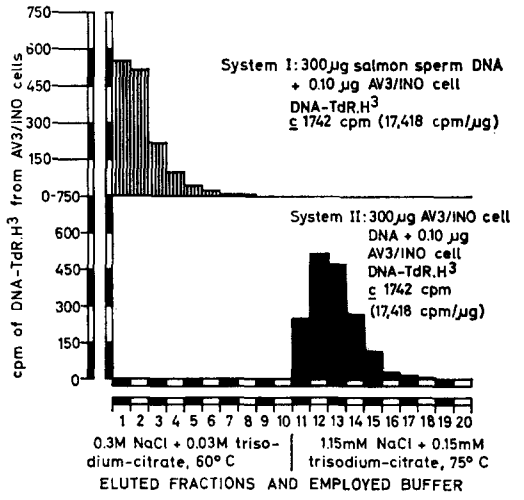

*Fig. 1b.*

tilled water. The exact amount of DNA and RNA used for each individual experiment will be appropriately indicated. We have also used the technique fixing mRNA instead of single-stranded DNA to agar particles.

The amount of nucleic acids fixed to the agar particles was calculated from the sample eluted with 5 M $NaClO_4$ as indicated. The intensity of hybridizing bonds was tested in the parallel experiment where samples had previously been treated with 0·3 M NaCl at 60°C, as it is known that under these conditions the partially or weakly hybridized offered nucleic acids become detached from the agar-fixed nucleic acids and are subsequently eluted with the 0·3 M NaCl and 0·03 M trisodium citrate buffer.

Results will be given as percentage of mated material, or as complementarity degree (C.D.) in parts per million (ppm), in order to make the data more easily comparable, considering the wide range of complementarity displayed by the different assayed systems.

DEMONSTRATION OF THE DEGREE OF COMPLEMENTARITY OF DNA BY THE PROCEDURE OF DUPLEX FORMATION UPON AGAR




The amount of radioactivity in the fractions eluted with 1.5 mM NaCl plus 0.15 mM trisodium citrate solution at 75° C indicates the degree of complementarity

*Fig.* 2.

*Radioactivity measurement*

Samples were prepared for liquid scintillation spectrometer.

Channels-ratio counting was chosen for singly labelled samples containing either $H^3$ or $P^{32}$ having relatively high counts. The counter gain was adjusted to obtain the highest counting efficiency, that was 45·7% for unquenched tritium standard. For $H^3$ samples having low counts, the ratio between the squares of the net count rate and the background rate was used instead the net count rate, and balance point counting realized.

For counting samples simultaneously labelled with $H^3$ and $P^{32}$ a two-channel counting procedure was followed.

## RESULTS

Figure 2 demonstrates the specificity of DNA-DNA hybridization between complementary strands. The employed systems are indicated in the figure. DNA-TdR·$H^3$ from AV3/INO-human amnion cells were eluted with the 0·3 M NaCl and 0·03 M tri-sodium citrate buffer at 60°C, indicating a practically complete absence of complementarity between AV3/INO DNA and salmon-sperm DNA (upper part of the graph). On the contrary, DNA-TdR·$H^3$ from human-amnion AV3/INO cells was fixed to agar-bound AV3/INO cell DNA and almost completely eluted in the complementary fractions (lower part of the graph).

In the complex experiment using doubly-labelled nucleic acids from AV3/INO-human amnion cells, represented in Fig. 3, the uridine-5·$H^3$-labelled RNAs correspond to the stable and long term labelled fractions (all along 6 days) while the $P^{32}$-labelled fractions are the unstable and short term labelled fractions (45 min labelling). For this experiment, 530 µg of DNA were fixed to 350 mg of 1% agar, and 51 µg rRNA-UR·$H^3$-$P^{32}$, 57 µg mRNA-UR·$H^3$-$P^{32}$ and 12·75 ug mRNA-UR·$H^3$-$P^{32}$ were passed through the column. Complementarity was evident for 0·52% UR·$H^3$-labelled mRNA, 86·5% $P^{32}$-labelled mRNA, 12·6% mRNA-UR·$H^3$, 4·5% mRNA-$P^{32}$, 1·2% rRNA-UR·$H^3$ and 0% $P^{32}$-RNA.

Figure 4 represents an experiment comparing the complementarity shown by AV3/INO-human amnion cell DNA and mRNA, when confronted with isogenic DNA. Only 10 µg of human-amnion DNA were fixed to 200 mg of 1% agar. As it is shown, increasing amounts of offered DNA-TdR·$H^3$ and mRNA-$P^{33}$ varying between 10 and 180 µg, gave increasing percentages of hybridization until a maximum of 89% for DNA-TdR·$H^3$ and 45·5% for mRNA-$P^{32}$ was reached. Similar results have been described by B. J. McCarthy and E. T. Bolton for complementarity between DNA and mRNA from Escherichia coli [6].

As Fig. 5 indicates, after DNA-mRNA-UR·$H^3$-$P^{32}$ hybridization, DNA still has binding sites for mRNA-UR·$H^3$-$P^{32}$ (9%) and after DNA saturation with mRNA and sRNA, rRNA-UR·$H^3$ is also bound (0·6%).

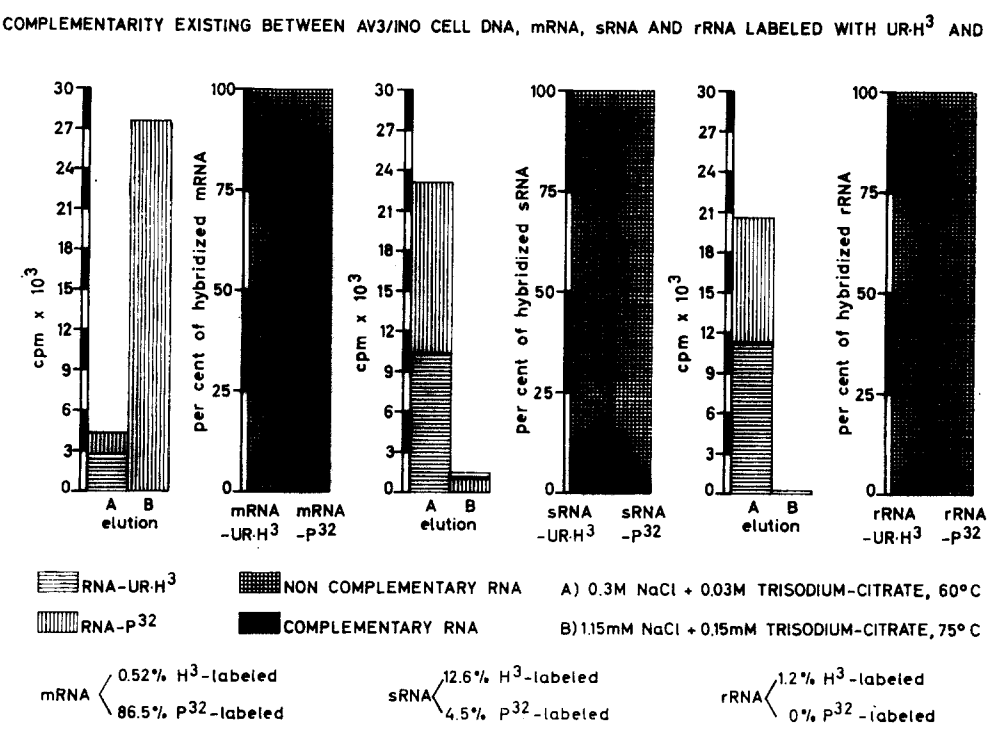



*Fig.* 3.

Isologous normal and cancer cell DNAs were confronted in the following systems (Fig. 6): (a) normal mouse embryo DNA with normal mouse embryo DNA-P$^{32}$ produced a complementarity of 90%; (b) CV virus-induced mouse tumour DNA with DNA-P$^{32}$ from the same tumour gave a complementarity of 87%.

The hybridization systems that led us to know about the "informational" differences existing between the mRNA-informing DNA fraction of normal and cancer cells are indicated in Fig. 7. Mouse embryo DNA confronted with a saturating amount of normal mouse embryo mRNA-P$^{32}$ and of CV virus-induced tumour mRNA-P$^{32}$ resulted respectively in 47·2% and 37·7% of complementary material. And CV virus-induced tumour agar-DNA received a saturating amount of CV virus-induced mouse tumour mRNA-P$^{32}$ and of normal embryo mRNA-P$^{32}$ registering for the respective system 46·5% and 31·2% complementary material.

Plotting of the results for virus-cell nucleic acids hybridization produced straight lines satisfying linear equations, as shown in Fig. 8.

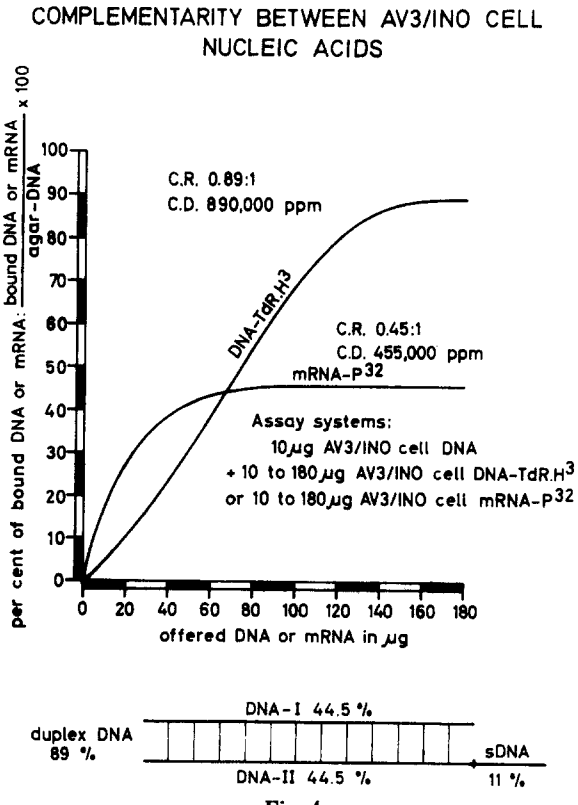



*Fig.* 4.

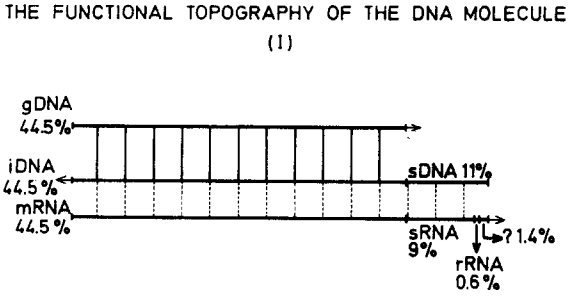



*Fig.* 5.

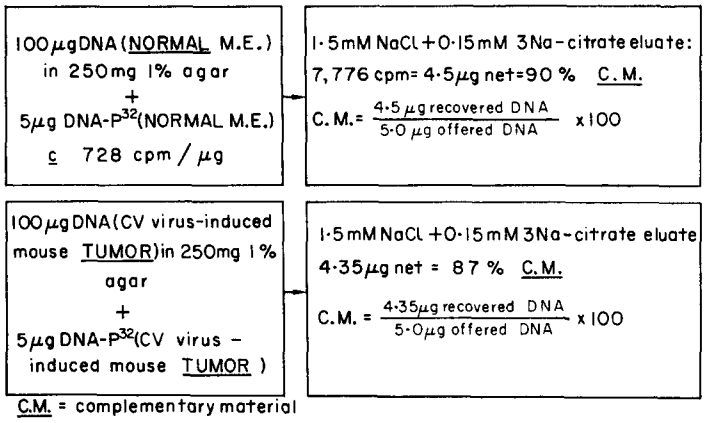



*Fig.* 6.

*System* 1 (*line* 2). CV virus-induced mouse tumour DNA was fixed to agar in amounts varying between 300 and 1500 μg, and the degree of mating with 1 μg of CV-virus DNA-TdR·$H^3$ having 11,200 cpm/μg observed. The results of this experiment showed the presence of hybridization between one part of CV-virus DNA and 41,000 parts of CV virus-induced mouse tumour DNA.

*System* II (*line* 3). The degree of complementarity between normal-mouse DNA and CV-virus DNA was searched for by mixing

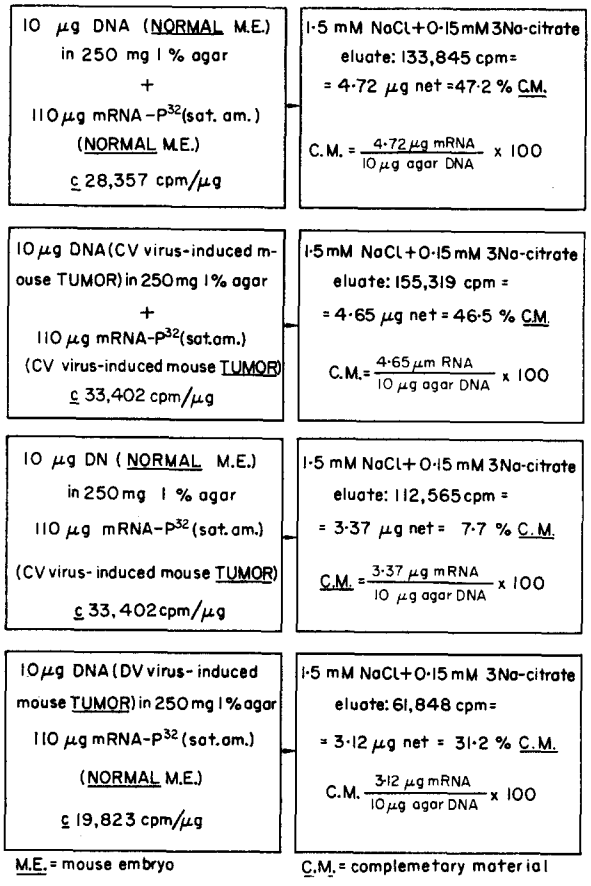



*Fig.* 7.

300–1500 μg of agar-fixed normal mouse DNA with 1 μg of CV-virus DNA-TdR·$H^3$ having 11,200 cpm/μg. Complementarity existed in one part of CV-virus DNA for each 111,000 of normal-mouse DNA.

*System* III (*line* 4). When amounts of normal-hamster DNA varying from 1000 to 2000 μg were fixed to agar and 1 μg of CV-virus DNA having 11,200 cpm/μg was added, complementarity was shown between one part of CV-virus DNA and 399,400 parts of normal-hamster DNA.

*System* IV. Amounts of 300–1500 μg of TC-CV/INO-cell line DNA were fixed to 1% agar, incubated with 1 μg of CV virus DNA-TdR·$H^3$ having 16,800 cpm, and the preparation transferred to a chromatography column. The plotting of CV-virus DNA in cpm and nanograms against the amount of TC-CV/INO-cell DNA in micrograms produced the usual straight line satisfying a linear equation showing complementarity of 1 part of virus DNA per 2340 parts of cell DNA.

*System* V (*line* 5). Confronting a DNA-agar with TC-SV40/INO-cell DNA varying from 300 up to 1500 μg, with samples containing 1 μg of CV virus DNA-TdR·$H^3$, complementarity corresponded to 1 part of viral DNA and 99,000 parts of cellular DNA.

*System* VI (*line* 6). To complete the data mentioned in this work, we studied the complementarity existing between human normal DNA and CV virus DNA. The hybridization system employed consisted in amounts of DNA from normal human amnion varying between 1000 and 3500 ng that were confronted with 1 pg of CV virus DNA-TdR·$H^3$ having 11,200 cpm/μg. Results, as indicated in Fig. 8, show that 1 part of CV virus DNA finds complementarity in 500,000 parts of normal-human amnion DNA.

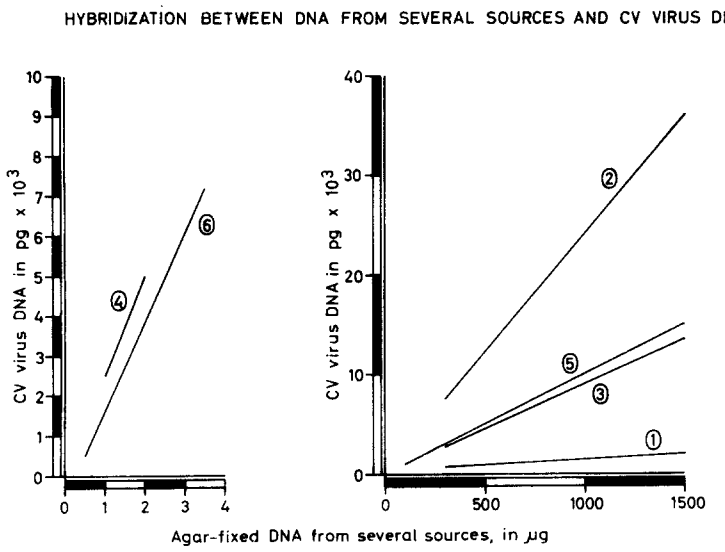



*Fig.* 8.

## DISCUSSION

Only one part of salmon-sperm DNA hybridized with 833,333 parts of AV3/INO-cell DNA, corresponding to 1·2 ppm, while for the human-amnion cells DNA-DNA system a ratio was obtained of 0·89:1 equivalent to 890,000 ppm (Fig. 2). The different proportion of hybridization between DNA samples if they belong to the same cell source or if they proceed from unrelated sources, indicates that the differences between the mating ratios can be interpreted as genetic similarity.

The data from the hybridization studies between cell DNA and doubly-labelled cell mRNA, sRNA and rRNA show: (a) that rRNA is the longest-living and slowest forming, and mRNA the shortest-living and the fastest-forming RNA, while sRNA occupies an intermediate position; and (b) that a large part of the length of the DNA chain is complementary to mRNA, while only a small region of it is complementary to sRNA and rRNA.

The results are practically the same whether mRNA, sRNA and rRNA are passed successively through the same agar-DNA column or through separate columns, so that the DNA complementary regions are independent and specific. Due to the big amount of cell rRNA, the only explanation is the existence of a few rRNA-synthetising cistrons corresponding to a small amount of molecular species of rRNA, that are serially produced in a concentrated way.

The different curves of complementarity shown by mRNA and DNA when hybridized with DNA from the same cell source (Fig. 4) indicate that: (a) isogenic-DNA mating takes place for 89% of the total length of the DNA molecule, while having a "singular" fragment (sDNA) comprising 11% of the DNA chain. We have registered similar results for other cell systems. The existence of native DNA existing in a single stranded state was described by us in human amnion cells, mouse embryos and mouse tumours as a part of the DNA macromolecule probably hybridized to RNA within the cell and complementary to sRNA and rRNA [7]. Simultaneously, Painter and Schaefer described the existence in HeLa cells of short single stranded segments having about 2000 nucleotides formed at the growing point during DNA replication [8]. (b) As for the 45·5% of mRNA hybridizing with DNA, it coincides with a complementarity between the complete population of mRNA molecules and about a half of the truly duplex DNA molecule showing DNA–DNA complementarity, a fraction that we call "informative" DNA strand or iDNA, calling "genetic" DNA strand or gDNA to the fraction which is complementary to iDNA.

The successive hybridization of DNA with mRNA-UR·$H^3$-$P^{32}$, sRNA-UR-$H^3$-$P^{32}$ and rRNA-UR·$H^3$-$P^{32}$ show that sRNA and rRNA are complementary to the apparently "singular" fraction of DNA or sDNA (Fig. 5).

The results obtained from the hybridization between islogous normal and cancer cell DNA, are similar to those obtained for AV3/INO-human amnion DNA-DNA systems, and reveal again that while most of the DNA single strands belong to a complementary duplex molecule, there exists a DNA region comprising between 10 and 13% of the length of the DNA strands as a "singular" strand of sDNA. If our results are accurate, cancer cell seems to

possess some "aberrant" forms of DNA which are responsible for the increasing in the sDNA fraction. Within the cell, sDNA probably exists as a natural hybrid DNA–DNA duplex. The topography of the DNA molecule indicates that it has a complex activity formed by a series of different functions that are carried out at a completely different rate. The megacistronic DNA forming the cell genome exerts its function as a cyclical rhythmic clock throughout the whole cell cycle, although the several zones display their activity at a different velocity. Recent experiments in our laboratory have led us to suspect that some parts of the megacistronic DNA cyclical rhythmic clock can be excluded by a short circuit during viral infections.

Let us see now the "informational" differences existing between the mRNA-informing DNA (iDNA) fraction of normal and cancer cells.

The realized experiments led us to the following conclusions (Fig. 9): (a) 47·2% of the normal mouse embryo DNA is complementary to mRNA from the same biological source; (b) 46·5% of cancer-cell DNA hybridizes with the total population of cancerous mRNA. These results mean that, in normal cells, all the mRNAs have a correspondence with cistrons of normal cell DNA on the iDNA strand. In the same manner, all the cancerous mRNA molecules are informed by complementary cistrons present in cancer cell DNA; (c) 33·7% of cancer cell mRNA hybridizes with normal-cell DNA, indicating a lack of mRNA information due to inhibition, disappearance or alteration of cistrons being present in normal cell; (d) 31·2% of normal-cell mRNA hybridizes with cancer-cell DNA, indicating that some cistrons informing normal mRNA are missing or altered in cancer-cell DNA and, in addition, that cancer cell contains mRNA molecules able to bind with cancer-cell DNA, but that find no specific sites for binding in normal-cell DNA.

In short, cancer-cell mRNA does not contain some types of mRNA existing in normal cell or else their production is blockaded, and possesses some mRNA species that can be considered as proper of the malignant cell.

If the totality of cistrons is transcribed to mRNA when this represents 47·2% of duplex DNA converted into single strands, then the existence of hybridization with 33·7% of single-stranded total DNA from normal cells corresponds to a lack, inhibition or alteration of 21·1% of the normal-cell cistrons. We do not know if all the cistrons can be interpreted as specific cistronic possibilities (potential-mRNA variations) or rather as existence of repeated cistrons, but at a given moment and for a particular cell type, not so many molecular species of mRNA seem to be produced. Considering the actual existence of about 30,000 types of mRNA in normal cell [g], cancer-cell iDNA in the studied system would conserve 21,419 actual messages that are common to both normal and cancer cells and lose 8500.

By the same token, as to the complementarity of only 31·2% of cancer-cell DNA with normal mRNA is concerned, it corresponds to the conservation of 19,831 mRNA species and the loss of 10,169, indicating that in cancer-cell DNA some of the cistrons present in normal-cell DNA are missing. Probably some cistrons having wild sense codons are converted by blockade, mutation or deletion into nonsense and missense codons destroying the activity of the whole cistrons, genes and operons, while, at the same time, cancer cell codifies a series of new genetic and "informational" properties.

On the other hand, hybridization between 500 μg of cancerous agar-DNA and 50 μg of cancerous rRNA or sRNA indicated a similar length (8·8%) of sRNA-informing DNA to that in the normal cells (9·0%), but a smaller amount of rRNA-informing DNA, occupying only 0·47% of the DNA length, probably at the sDNA region.

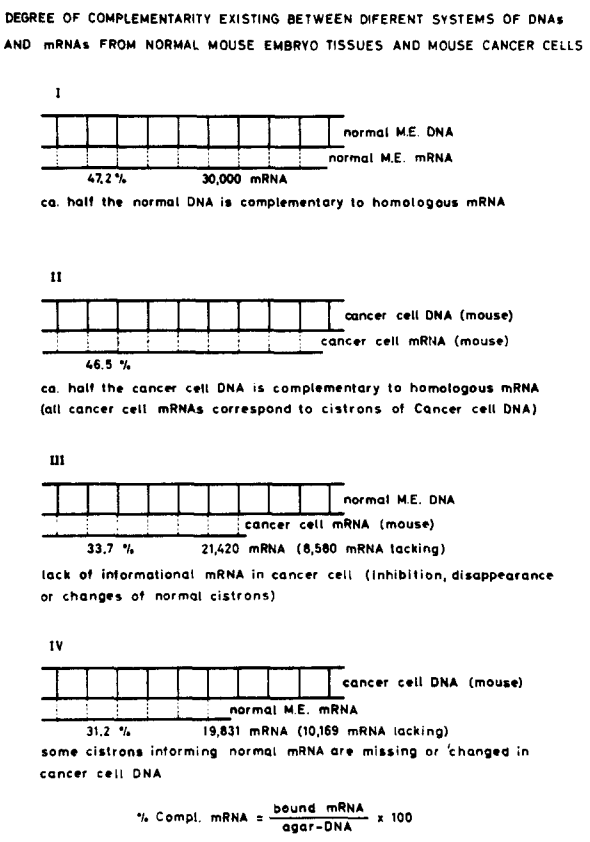



*Fig.* 9.

When CV-virus DNA was confronted with CV virus-induced tumour DNA, hybridization was found to be 1:41,000. Considering the facts that the mating of CV-virus DNA and normal mouse DNA corresponded to a complementarity ratio of 1:111,000, and that CV-virus DNA is bound with normal-hamster DNA in a proportion of 1 to 399,400, we can make the following conclusions.

The degree of complementarity indicates similar parts of the corresponding genomes. If, as previously said, single-stranded DNA samples bound in a proportion of 1 to 833,333 when they proceeded from unrelated sources, then the differences between the mating ratios can be interpreted as genetic similarity. This was evident when we observed that CV-virus DNA obtained from mouse-embryo primary cultures showed a higher degree of complementarity with normal-mouse DNA (1:111,000) than either with normal-hamster DNA (1:399,400) or with normal human-amnion DNA (1:500,000). The results registered after confrontation of CV virus-induced mouse tumour, corresponding to a mating ratio of 1:41,000, indicated that the cell genome of the transformed cell contained parts of the viral genome or they are represented in cell genome in some way. In addition, while normal-hamster DNA hybridized with CV-virus DNA maintained in mouse-embryo primary tissue cultures in a proportion 1 to 399,400, the mating ratio increased to 1:99,000 when a hamster-established line transformed by SV40 virus, and designated as TC-SV40/INO line was employed. This can be considered as the existence of some common parts in the genome of the transformed cells whether they were made neoplastic by CV virus or by SV40 virus.

DNA extracted from CV virus growing in mouse-embryo primary cultures, when tested against TC-CV/INO-cell DNA, showed a complementarity ratio indicating that one out of 2340 DNA fractions of the cell genome in TC-CV/INO cell was complementary to the viral genome.

The complementarity of DNA extracted from a CV virus-induced hamster tumour with CV-virus DNA was 17·5 times greater than that existing between DNA isolated from a CV virus-induced mouse tumour and CV virus DNA. When both virus-cell DNA systems are compared, the complementarity ratio found for TC-CV/INO DNA+CV-virus DNA is rather high. This is due to the fact that the mouse tumour does not liberate any virus, the complementarity revealing viral DNA incorporated in some way to the cell genome or cellular genetic information induced by the virus. As for the hamster tumour, we attribute the results to the existence of viral DNA, extracted simultaneously to the cell DNA. Actually, the virus titer of the TC-CV/INO-cell cultures used in the experiments was found to be a TCID50 of $10^{3 \cdot 5}$.

In a former work [10] we have summed up conjointly the results obtained in our experiments. We can only recall here that the confrontation of two complete populations of homologous single-stranded DNA afforded a complementarity degree of 890,000 ppm, while mRNA face to face with DNA from the same cell source gave 460,000 ppm. Complementarity degree for CV-virus DNA *vis-à-vis* with virus-free CV virus-induced mouse tumour DNA was 24 ppm. For the other studied systems, complementarity for CV virus DNA was 10 ppm when confronted with TC-SV40/INO cell DNA, 9 ppm with normal-mouse DNA, 2·5 ppm with normal-hamster DNA, 2 ppm for normal human amnion, and 1·2 ppm for salmon-sperm DNA.

The main conclusions of the reported experiments are the following: 1. Viral genome is represented in some way in the genome of the normal susceptible cell and to a larger extent in the transformed cell. 2. There are some common parts in the genomes of the transformed cells, whether they are mouse cells made neoplastic by CV virus or hamster cells converted into malignant by SV40 virus. 3. In addition to common changes produced by different carcinogenic viruses on cell DNA, each virus is specifically represented in cell cistrons.

From the reasons explained elsewhere [11] and in order to make easier the calculations, we took 2 ppm as a rough equivalent to one gene when single-stranded DNA made out of duplex DNA is paired, and 1 ppm as equivalent to one informational cistron when single-stranded mRNA is confronted. Data written in previous reports [10, 11] were given taking these considerations into account. Further experiments, however, indicate that virus DNA seems to be an exception, because both viral single strands resulting from duplex DNA denaturation appear to hybridize along the gDNA strand, so that in this case, parts per million of complementarity correspond to the same number of cistrons in cell DNA, without detriment of the obliged fact that the iDNA region complementary to the gDNA portion which is in turn complementary to one of the viral-DNA strands, indicated in Fig. 10 as vDNA-I, has to possess the same base sequence

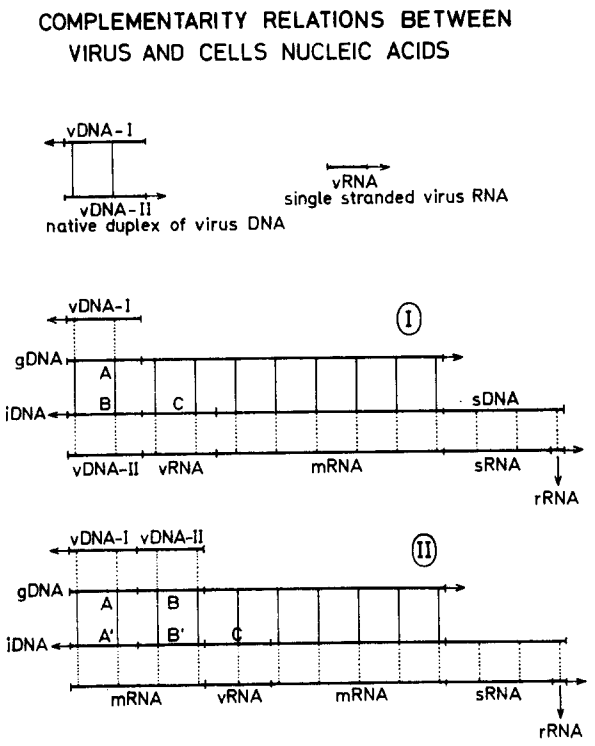



*Fig.* 10.

as the other viral-DNA strand (vDNA-II), and vice versa that the gDNA part complementary to vDNA-II has a complementary part along iDNA reproducing the base sequence of vDNA-I. This indicates that during infection with DNA viruses, cells produce the complementary iDNA strand to synthesize specific mRNA. Our experiments do not indicate whether the gDNA regions complementarity to vDNA-I and vDNA-II are situated one after another or separated by other cell cistrons.

Taking these facts into consideration CV virus DNA has 4 genes in common with susceptible uninfected cells, while it possesses 12 genes in common with CV virus-transformed cells, transformation implicating 8 additional genes.

However, DNA from CV virus adapted to mouse cell seems to have one gene in common with normal-hamster DNA and 5 genes in common with an SV40-induced hamster tumour established *in vitro* (TC-SV40/INO cell line). The CV virus-transformed cell having 8 genes related with malignant conversion, 4

| Nucleic acids | Characteristics | Normal cell | Cancer cell |
|---|---|---|---|
| Native duplex DNA | Megacistronic genome: gDNA:iDNA-sDNA:RNA complex | 450,000 genes | 443,320 genes |
| gDNA | iDNA-complementary DNA | 450,000 genetic units | 443,320 genetic units |
| iDNA | gDNA-complementary DNA; at the same time mRNA-complementary DNA | 450,000 cistrons | 443,320 cistrons |
| sDNA | sRNA- and rRNA-complementary DNA forming natural RNA-DNA hybrids | 110,000 cistrons | 130,000 cistrons |
| mRNA-informing DNA | At iDNA; potential mRNA messages (specific cistronic possibilities or repeated cistrons?) | 450,000 cistrons | 443,320 cistrons |
| mRNA | Actually formed mRNAs | 30,000 species | 29,555 species |
| sRNA-informing DNA | At sDNA; forming sRNA-DNA hybrids; potential sRNAs (specific cistronic possibilities or repeated cistrons?) | 90,000 cistrons | 88,000 cistrons |
| sRNA | Actually formed sRNAs | 6000 species | 6000 species |
| rRNA-informing DNA | At sDNA; forming natural rRNA-sDNA hybrids; potential rRNAs (specific cistronic possibilities or repeated cistrons?) | 6000 cistrons | 4700 cistrons |
| rRNA | Actually formed rRNAs | 400 species | 314 species |

Note. Data refer to nucleic acids from human and mouse cells.

*Fig.* 11. *Functional distribution of DNAs and RNAs in normal and virus-transformed cancer cells based on nucleic acids complimentarity.*

| | | |
|---|---|---|
| I. Normal cell rDNA | (a) | Contains information for:<br>30,000 normal-cell mRNAs;<br>21,420 cancer-cell mRNAs, that are common to both normal and cancer cell. |
| | (b) | Does not contain information for:<br>8580 cancer-cell mRNAs, that are specifically cancerous. |
| II. Cancer cell iDNA | (a) | Contains information for:<br>29,555 cancer-cell mRNAs;<br>19,831 normal-cell mRNAs, that are common to both normal and cancer cell. |
| | (b) | Does not contain information for:<br>10,169 normal-cell mRNAs (that is, cancer cell lacks this number of normal mRNAs). |

Note. Data refer to mouse cells nucleic acids.

*Fig.* 12. *Information for mRNA synthesis contained in normal and cancer-cell DNA.*

genes seem to be identical for the CV virus-induced mouse tumor and the SV40 virus-induced hamster tumor investigated.

By combining all the data given in this paper, part of which appeared elsewhere [10, 11], we prepared the tables of Figs. 11–13. The number of genes in mammalian cells has been calculated very diversely by the different authors, the most conservative giving about 50,000 [12, 13] or 100,000 [14], and the most exaggerated about 1,000,000. In the alternative of having to choose, we took the middle, just for practical reasons, as it coincided with some numbers of our experiments. Consequently, the numbers given in these tables do not claim to be exact figures, but rather relative values when arbitrarily considering a type normal cell to have 450,000 genes, just to make easier the calculations according to our experimental results.

| Characteristics | Normal cells | Cancer cells |
|---|---|---|
| Average number of normal mRNAs present | 30,000 | 20,625 |
| Average number of normal mRNAs lost | 0 | 10,169 |
| Average number of cancer-specific neoformed mRNAs (mRNAs linked to the cancerous character as such) | 0 | 8580 |
| CV-virus genetic units present in normal susceptible cells (genetic units common to both, normal-cell gDNA and virus DNA): | | |
| (a) mouse cells | 9 | |
| (b) hamster cells | 2·5 | |
| (c) human amnion cells | 2 | |
| Additional CV-virus genetic units in the gDNA of the transformed cells | | 15 |
| Genetic units common to TC-SV40/INO (an SV40 virus-induced hamster tumor established *in vitro*) and CV virus-induced mouse tumor | | 7 |
| Total virus-genetic units existing in the gDNA of CV virus-transformed cells | | 21 |
| mRNAs linked to the cancerous character of viral origin (used for viral synthesis in vegetative reproduction or for the metabolic pathway related to viral DNA in lysogenic cells) | | 31 |

Notes. (1) We maintain CV virus by successive passages in mouse embryo primary tissue cultures and in subcultures of our TC-CV/INO established cell line, which is formed by virus-releaser cells originally derived from a CV virus-promoted hamster tumor established *in vitro*. All experiments referred here were done using CV virus maintained in mouse-embryo cells.
(2) Data refer to mouse-cells nucleic acids, unless otherwise indicated.

*Fig.* 13. *Common and specific mRNAs of normal and cancer cells, and viral genes present in normal and cancer cells.*

## SUMMARY

*Hybridization between isologous nucleic acids has shown that complementarity exists for* 89% *of the single-stranded DNA; this duplex DNA is formed for* 44·5% *by a genetic-DNA strand (gDNA), and for* 44·5% *by an informational-DNA strand (iDNA). The remaining* 11% *is constituted by a "singular"-DNA strand (sDNA). Duplex DNA is formed by the pairing of gDNA-iDNA, and sDNA exists within the cell as a natural-DNA–RNA hybrid duplex. The informational strand is associated to mRNA biosynthesis, representing* 44·5% *of the DNA and the complete population of mRNA, while sDNA is associated* 9% *to sRNA and* 0·6% *to rRNA.*

*Cancer cell lacks some information of cistrons existing in normal cell and has some additional information not present in normal cell. The amount of sRNA-informing DNA seems to be the same for normal and cancer cells, but rRNA-informing DNA is apparently diminished in cancer cell.*

*Viral genome is represented in some way in the transformed cell. There exist some common parts in the genomes of transformed cells whether they are mouse cells made neoplastic by CV virus or hamster cells converted into malignant by SV*40 *virus. However, in addition to common changes produced by different carcinogenic viruses on cell DNA, each virus is specifically represented in cell cistrons.*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# Letter to the Editor

## Effect of Insulin and of Alloxan Diabetes on Growth of the Rat Mammary Carcinoma *in vivo**

J. C. HEUSON and N. LEGROS

*Laboratory of Clinical Investigation, Service of Internal Medicine, Institut Jules Bordet (Université Libre de Bruxelles), 1, rue Héger-Bordet, 1000-Brussels, Belgium*

As previously reported, a majority of dimethylbenz(a)anthracene (DMBA)-induced rat mammary carcinoma are strongly dependent on insulin for cell proliferation in organ culture, although about one third of all randomly-selected tumors are insulin-independent and freely proliferate in insulin-free medium [1].

Investigations on the mechanism of this growth-promoting effect of insulin led us to believe that it was not merely a permissive effect, bearing on, for example, energy-yielding reactions [2]. Rather it appeared to be a process more directly related to DNA synthesis, involving activation or induction of such enzymes as the soluble DNA polymerase [3]. It seemed therefore conceivable that insulin might also influence growth of the mammary tumors *in vivo*. In order to test this hypothesis, tumor-bearing rats were either treated with large doses of insulin or submitted to alloxan diabetes.

Mammary carcinoma were induced in female Sprague-Dawley rats by a single feeding of DMBA at age 50 days.

The insulin experiment was started 107 days after carcinogen administration. Rats were matched in lots of 4 with respect to number and size of their tumors; they were then randomized into 4 groups of 17. The experimental group received subcutaneous injections of Lente insulin (Novo), 2·5 U per 100 g body weight, daily except on Sundays. They were given a 10% glucose solution as drinking fluid, which they consumed in large amounts, in order to reduce the risk of lethal hypoglycemia. The control group received saline injections and plain water as drinking fluid. Two additional groups received, as only treatment, either a 10% glucose beverage or insulin injections. The size of the tumors was calculated as "surface" by multiplying two transverse diameters. All results were expressed as total tumor surfaces (sum of individual surfaces) per rat.

The results are given in Fig. 1. Administration of insulin together with the glucose beverage (D) produced an 8·3-fold increase in tumor growth (average increment of total tumor surface per rat) during the experimental period of 6 weeks. Administration of the glucose solution alone (B) or of insulin alone (C) also markedly stimulated tumor growth, but significantly less than insulin plus glucose.

Diabetes was induced in 16 tumor-bearing rats, 146 days after DMBA carcinogenesis, by means of a single administration of alloxan, followed by a two-week insulin replacement therapy aimed at reducing mortality. Twelve rats survived during the 6-week period of observation. Ninety per cent of the 57 tumors present at onset of the experiment regressed

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rapidly. This is shown on the graph of Fig. 2, where day 0 is the day following the last insulin injection and the curves represent the total surface per rat of the regressing tumors. Only 5 tumors (not represented on the graph) remained unchanged or increased in size. Eight such tumors, growing in diabetic rats, were tested in organ culture and found little or not at all insulin dependent.

These data clearly demonstrate that large doses of insulin markedly stimulate growth of the rat mammary carcinoma, whereas suppres-

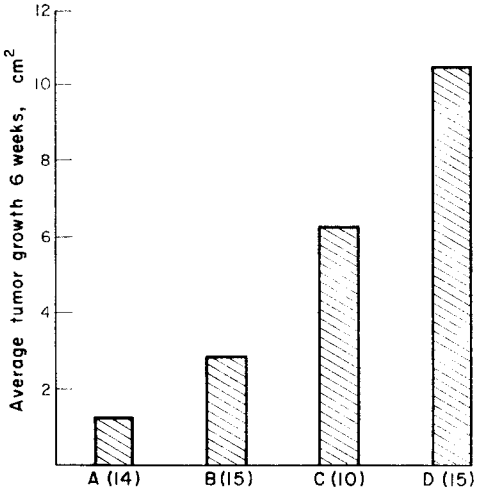


*Fig. 1. Effect of insulin (2·5 U per 100 g body weight) and/or 10% glucose solution as drinking fluid on growth of rat mammary carcinoma. A: control group. B: 10% glucose only. C: insulin only. D: insulin plus 10% glucose. The figures in parentheses represent the number of rats still living and healthy in each group at the end of the experimental period. Statistical significance (Student's t test) of differences between matched groups: B—A, $p<0·01$; C—A, $p<0·02$; D—A, $p<0·001$; D—B, $p<0·001$; D—C, $p<0·05$.*

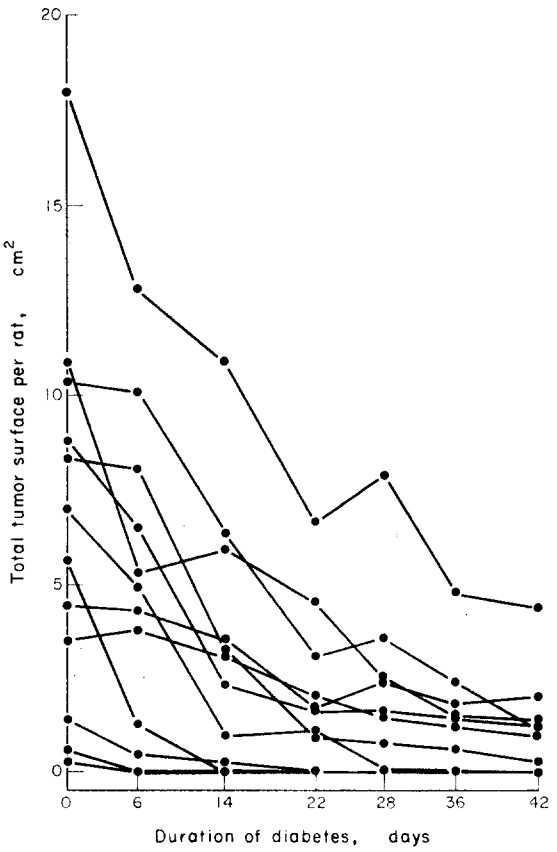


*Fig. 2. Effect of alloxan diabetes on rat mammary carcinoma. Each curve represents the total surface per rat of the regressing tumors (see text).*

sion of the endogenous insulin produces their regression, except in rare cases of tumors that are also insulin-independent *in vitro*.

It is thought that the growth-regulating effect of insulin demonstrated here may reflect a direct action of insulin on the tumors *in vivo*. This is suggested by the results of the organ culture experiments referred to above. It may be argued that the insulin-induced hypoglycemia may stimulate secretion of pituitary growth hormone which in turn may stimulate tumor growth. Against this interpretation, Pearson [4] reported that growth hormone, unlike prolactin, was unable to reactivate growth of regressed mammary tumors after hypophysectomy. In addition, the glucose beverage which, alone or in combination with insulin, stimulated tumor growth in our experiments, may be expected to increase insulin production but also to depress growth hormone secretion.

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# Growth Inhibition of Rat Mammary Carcinoma and Endocrine Changes Produced by 2-Br-α-Ergocryptine, a Suppressor of Lactation and Nidation*

J. C. HEUSON, C. WAELBROECK-VAN GAVER and N. LEGROS

*Service of Internal Medicine and Laboratory of Clinical Investigation†, and Laboratory of Cytology and Experimental Oncology, Institut J. Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, Brussels, Belgium*

PROLACTIN plays a major part in the induction and growth of rat mammary tumors [1, 2]. Pearson *et al.* [2], studying the 7,12-dimethylbenz(a)anthracene(DMBA)-induced mammary carcinoma of the Sprague–Dawley rat, reported that prolactin reactivates tumor growth after ablation of the ovaries, adrenals and pituitary gland. Moreover, administration of perphenazin, which stimulates prolactin secretion, enhances the incidence and growth of the tumors, even after oophorectomy [3]. Finally, administration of antibody against prolactin induced tumor regression [4].

The present work studies the effect of 2-Br-α-ergocryptine (CB-154, Sandoz Ltd.) on the formation and growth of the DMBA-induced rat mammary carcinoma. This derivative of α-ergocryptine, an alkaloid of ergot, displays properties similar to those of the parent substance as well as of ergocornine. All three compounds are potent inhibitors of nidation and lactation in the rat, probably through interference with prolactin secretion [5, 6], although there is evidence suggesting that more than a single mechanism may be involved [6]. In view of these considerations, it was expected that administration of CB-154 might inhibit tumor growth and decrease the number of newly formed tumors in the rat.

## MATERIAL AND METHODS

Mammary carcinomas were induced by a single feeding of DMBA to female Sprague–Dawley rats at the age of about 50 days. Thirteen to 16 weeks thereafter, they were examined for number and size (expressed as "area"= product of two transverse diameters) of the tumors. They were then match-paired according to these criteria and divided at random into two groups of 16. One group received s.c. injections of CB-154 dissolved in 20% DMSO, at a daily dose (6 days/week) of 3 mg/kg for 3 weeks and of 6 mg/kg for an additional 3-week period. The control group received only the solvent.

## RESULTS

Fifteen pairs were suitable for evaluation at the end of the experimental period (one pair was rejected because the rat of the control group lost more than 10% of its weight due to intercurrent disease). The results are shown in Table 1. In the treated group 18 of the 27 tumors originally present decreased in size while 9 increased or remained unchanged; only 16 newly-formed tumors appeared. In the control group, 8 tumors decreased in size, 19 increased or remained unchanged and 32

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*Table 1. Effect of 2-Br-α-ergocryptine on rat-mammary tumor growth and on appearance of newly-formed tumors*

| Treatment | Number of tumors* | | | Average tumor growth per rat ($cm^2$) | Inhibition of tumor growth (%) |
|---|---|---|---|---|---|
| | Regressing | Progressing or unchanged | Newly formed | | |
| 2-Br-α-ergocryptine | 18 | 9 | 16 | 1·47 | 67† |
| Control | 8 | 19 | 32 | 4·49 | |

Rats were match-paired according to number and size of tumors and divided at random into 2 groups of 16, one group receiving the treatment and the other serving as control. Fifteen pairs were suitable for evaluation. The effect of treatment was analyzed in two ways: first with respect to the change in size of individual tumors and appearance of new ones; secondly, in regard to the change in total tumor area per rat (tumor growth), during the 6-week period of treatment.

*The inhibitory effect of treatment is significant, $P<0·01$ (Wilcoxon test; for detail on statistical calculation, see text).

†The inhibition of tumor growth is significant, $P<0·05$ (Wilcoxon test).

were newly formed. Thus the treatment increased the number of regressing tumors and decreased the incidence of newly formed tumors. This effect of treatment is statistically significant. This was demonstrated by ascribing a score to each rat. The score was calculated by giving the value minus one to each tumor which regressed, and plus one to each tumor which progressed, remained unchanged or was newly formed. The calculation was based on the assumption that the former type of tumor had the inverse biological meaning of the latter types. The Wilcoxon test, applied to the differences of scores within pairs, yielded $P<0·01$. On the other hand, the inhibitory effect of the treatment was demonstrated by comparing the change in total tumor area per rat during the 6-week period of observation (tumor growth per rat). The mean tumor growth was 1·47 $cm^2$ in the treated group and 4·49 $cm^2$ in the control group. This represents a 67%-growth inhibition due to the treatment, which is statistically significant (Wilcoxon test, $P<0·05$). CB-154 produced no change in weight nor visible side effect.

The preliminary results of an endocrine study of the effects of CB-154 are the following. The estrus cycle was determined on vaginal smears obtained daily during the last 9 days of the experimental period. In the treated group the estrus cycle was uniformly of 4 days in all rats. In the control group it was of 4 days in only four rats and of 5 days or longer in all others. CB-154 thus effects a highly significant shortening of the estrus cycle (Fisher's formula, $P<0·002$). At the end of the experimental

*Table 2. Effect of administration of 2-Br-α-ergocryptine for 6 weeks on the average weight of ovaries and number of corpora lutea per ovary*

| Treatment | Weight of 2 ovaries (mg) (average per rat) Day of estrus | | | Number of corpora lutea* (average per rat) Day of estrus | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 0 | 1 | 2 |
| 2-Br-α-ergocryptine† | 217·1 | 161·3 | 178·6 | 38·7‡ | 33·5 | 33·5 |
| Control | 64·5 | 79·4 | 67·1 | 14·0 | 16·5 | 17·5 |

The number of corpora lutea per ovary was estimated by counting them on a histological section cut at the level of maximal width; this simplified method yields a definite underestimation of the true value. Measurements were carried out on lots of four rats on the day of estrus (day 0) and on day 1 and 2 after estrus.

*In the treated group corpora lutea were of normal size or slightly enlarged. In the control group, they exhibited all stages of normal involution.

†All differences between the treated and control groups were highly significant. Weight of ovaries, $P<0·001$. Number of corpora lutea, $P\leqslant 0·05$ (Student's *t*-test).

‡Average for three rats.

period, lots of four rats from the treated and from the control groups were killed respectively on the day of the estrus, and on day 1 and 2 after estrus. Autopsy disclosed an unexpected finding: the ovaries of the treated group were markedly enlarged (Table 2). They contained a large number of corpora lutea, that were normal in size or slightly enlarged. On histological examination, about two thirds of these corpora lutea exhibited a normal appearance, being composed of typical round cells, whereas in the other third most cells were somewhat spindle-shaped and displayed an unusual overall whirling arrangement. None of the corpora lutea showed any sign of involution. In the control rats the ovaries had a normal histological appearance with corpora lutea in all stages of involution. Histological examination of the vagina from the treated and control animals disclosed the characteristic aspects of the corresponding phase of the cycle. At no time was there any sign of increased progestational activity. Treated and untreated specimens appeared identical. Contrasting with the marked changes observed in the ovaries, there was no significant effect of CB-154 on the weight of the adrenals and pituitary glands.

## DISCUSSION

Interpretation of these data is made somewhat difficult by the lack of determination of the serum and pituitary prolactin concentrations. However, it can be inferred from the known inhibitory effect of CB-154 on lactation in the rat [6] and from the general consensus about the inhibitory property of the parent compound, ergocornine, on prolactin secretion [5] that CB-154 in the present experiment probably inhibited prolactin secretion and thereby produced its inhibitory effect on mammary tumor growth. A most remarkable finding was the accumulation of a huge number of uninvoluted corpora lutea, some of them exhibiting unusual histological features, as a result of CB-154 administration. There is here concordant evidence suggesting that these corpora lutea were not functional and that, despite their considerable increase in number, they did not induce progestational effects. The maintenance of cycling itself, the shortening of the estrus cycles and the lack of increased mucification of the vaginal mucosa strongly support this view. A possible explanation, which is admittedly hypothetical, is that CB-154 interferes with the normal maturation and function of the corpora lutea, either by inhibiting prolactin secretion or by a direct effect on the luteal cells or both, and that this interference results in their inability to undergo normal involution and their consequent abnormal maintenance and accumulation.

In conclusion, it is suggested that CB-154 inhibits the development and growth of the DMBA-induced mammary carcinoma of the rat by selective interference with prolactin secretion, with paradoxical accumulation of inactive corpora lutea. Although the mechanism involved requires further elucidation, it may be said that, to our knowledge, administration of CB-154 is the only endocrine-related treatment which inhibits the growth of the rat mammary carcinoma without suppressing the estrus cycle.

Since the rat mammary carcinoma exhibits properties of hormone dependence resembling those of the human breast cancer, this substance might be of great interest in the treatment of the human breast cancer.

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## SUMMARY

*Administration of 2-Br-α-ergocryptine for 6 weeks to Sprague–Dawley rats bearing DMBA-induced mammary carcinomas increased the number of regressing tumors and decreased the incidence of newly-formed ones. This inhibitory effect on tumor growth was highly significant. In addition, the drug produced the following endocrine changes: shortening of the estrus cycle and accumulation of large numbers of corpora lutea, which seemed inactive with respect to secretion of progestational compounds and did not undergo normal involution. It is suggested that these effects, both on tumor growth and on endocrine functions, probably resulted from interference with prolactin secretion.*

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# The Development of "Spontaneous" Neoplastic Transformation *in vitro* of Cells From Young and Old Mice

L. M. FRANKS and S. HENZELL

*Department of Cellular Pathology, Imperial Cancer Research Fund, Lincoln's Inn Fields, London W.C.2, England*

THE ABILITY of some mammalian cells to produce malignant tumours when retransplanted into animals after cultivation *in vitro* [1, 2] should provide a useful system for testing the tumour-producing potential of cells from organs of animals of different ages. It was hoped that a series of experiments using this system might help to explain the age-associated differences in the incidence of tumours in different organs. Several factors thought to influence the development of neoplasia *in vitro* were also investigated. The ultrastructure of the cells was also examined and the results will be described in a later paper [3].

## MATERIAL AND METHODS

*Mice* of three different age groups from two inbred strains — C57BL($a^t$) Icrf and C3H (Heston and Bittner sublines) — from our colony of ageing mice were used. The age groups were embryo (18 days), young (3–20 days) and old (28–34 months).

*Tissue-culture methods*

Tissues were removed aseptically and rinsed in tissue-culture medium. *Explant cultures* (1–$2^3$ mm explants) were prepared by chopping with scalpels or a mechanical chopper. *Cell suspensions* were prepared from chopped tissue by trypsinization at 36·5°C in a fluted 250-ml Erlenmeyer flask. The trypsin (Tryptar, Armour) was used at a concentration of 500 units/ml in Ca.Mg free phosphate buffered saline [4], with about 4 parts by volume of solution to 1 part of chopped tissue. The cell suspension was removed when the supernatant became turbid and fresh trypsin was added. This usually took 2–5 min with embryonic and young tissue and up to 20 min for tissue from old animals. The first batch of supernatant was removed after 2–3 min and discarded. Subsequent batches were pooled after re-suspending in tissue-culture medium. The maximum period of trypsinisation was 4 hr. Cultures were set up in 225 ml baby's Pyrex feeding bottles. About 15 explants per bottle were used for explant cultures and about 800,000 cells from cell suspensions.

*The medium* used was Waymouth's medium 752/1 (5) with 10% calf serum. The serum concentration was reduced to 5% when cultures regularly formed monolayers in 7 days or less. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to primary cultures from old tissue only. Antibiotics were not used in subsequent cultures. Ten ml of medium was used for each bottle and was changed at weekly intervals as a rule, depending on growth and pH changes. Cultures were gassed with 5% $CO_2$ in air after each medium change and kept at 36·5°C. Cells were transferred only when a complete monolayer had formed. They were removed from the bottles by syringing, versene or trypsin (see Tables 1 and 2 for details). In the early transfers, when growth was slow, cells were transferred from one bottle into two. When the growth rate increased three bottles were inoculated. One bottle was used to maintain the cell line and the remaining two

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*Table* 1. *Tumour lines*

| Expt. no. | organ | Mouse strain | age | Culture methods Initial | Transfer | Slow growth phase (days) | Mouse implantation Days after culture initiated No tumour | Tumour |
|---|---|---|---|---|---|---|---|---|
| CBM 17 | Kidney | C57 | 3 days | T | T | 143(2) | | *283(23) |
| CBM 18N | Kidney | C3H | 3 days | T | T | 111(7) | | 246(28) |
| CBM 19 | Lung | C57 | 3 days | E | TVS | 110(7) | 225, 388, 402, 465 | 521(58) |
| CBM 25 | Kidney | C57 | 3 days | E | TS | 99(5) | | 286(22) |
| CBM 26 | Kidney | C57 | 3 days | T | T | 131(4) | | 204(12) |
| CBM 31 | Bladder | C3H | 5 days | E | S | — | | 205(3) |
| COM 1 | Heart | C57 | 29 mths | E | S | 209(6) | 209, 273, 322, 415 482, 488 | 638(58) |
| COM 2 | Lung | C57 | 28 mths | T | S | 177(3) | 174 | 229(9) |
| COM 3 | Prostate | C3H | 30 mths | E | S | 172(7) | 181, 221, 372 | 431(30) |
| COM 4 | Kidney | C57 | 34 mths | T | S | 99(3) | 128, 135 | 190(15) |
| COM 4 | Bladder | C57 | 34 mths | E | S | 199(5) | 201, 243, 321 | 388(23) |
| COM 5 | Tongue | C57 | 28 mths | E | S | 174(7) | 152, 181 | 243(18) |
| COM 6 | Brain | C3H | 29 mths | T | T | 180(3) | | 195(4) |
| COM 6 | Nerve | C3H | 29 mths | E | TS | 245(7) | 208, 276 | 358(17) |
| COM 6 | Spinal cord | C3H | 29 mths | E | S | 216(13) | 62, 168 | 208(11) |
| COM 6 | Tongue | C3H | 29 mths | E | S | 186(3) | 181, 274 | 334(18) |

Figures in parenthesis are transfer generations *in vitro*.
E = Explant.
T = Trypsin.
S = Syringing.
V = Versene.
* = Eye transplant.

at each generation were used either for animal inoculation, electron microscopy, frozen for storage in liquid nitrogen or discarded.

*Animal inoculations* were made using syngeneic mice. Concentrated cell suspensions from one bottle were injected subcutaneously into two or three mice (approximately $3 \times 10^6$ cells/mouse). In one experiment cells from 14 different lines were inoculated intraperitoneally, at a similar concentration. In a few cases a smaller number of cells were inoculated into the anterior chamber of the eye. Tumours which developed were transplanted and portions were stored in liquid nitrogen.

## RESULTS

One cell line was established from embryo-mouse tissue, 14 from young mice and 21 from old mice. The three stages in the establishment of cell lines — initial proliferative phase, "stationary" phase and recovery phase — described by other workers e.g. [6, 7, 8] occurred in these cultures but under our culture conditions the short initial proliferative stage could not be accurately measured. The period from the initiation of the culture until monolayers were regularly formed within 7 days was defined as the "slow growth phase" and thus includes the early proliferative phase and the "stationary" phase, when growth is sluggish. Details of the initial treatment, method of transfer, length of slow growth phase, and tumour production are given in Tables 1, 2 and 3. Cell lines with the same experiment number were derived from different organs from one animal, e.g. COM 3 lung, COM 3 kidney, COM 3 prostate. The cell lines prefixed CBM 18 are both derived from a common pool of cells from trypsinised young mouse kidney. CBM 18S was routinely transferred by syringing and was not exposed to trypsin after the initial preparation of the cell suspension. CBM 18N was routinely transferred by trypsinisation. Tumours developed after subcutaneous or intraocular inoculation of cells from six young and 10 old cell lines (Table 3). They were of spindle and giant cell type. The ultrastructure, histochemistry and transplantation behaviour of these tumours will be described in detail in a later paper.

*Table 2. Non-tumour lines*

| Expt. no. | tissue | Mouse strain | age | Culture methods Initial | Transfer | Slow growth phase (days) | Mouse implantation Days after culture initiated No tumour |
|---|---|---|---|---|---|---|---|
| CEM | 1 (Embryo) | C57 | 18 day embryo | E | VTS | 122(6) | *232, 322(24) |
| CBM | 15 Kidney | C57 | 3 days | T | T | 172(1) | 297, 398(25) |
| CBM | 18S Kidney | C3H | 3 days | T | S | 80(4) | *114, 216, 317, 355, 672(42) |
| CBM | 23 Lung | C3H | 20 days | E | TVS | 83(4) | 218, 303(27) |
| CBM | 29 Heart | C3H | 7 days | E | T | 57(4) | *182, 230(25) |
| CBM | 29 Spleen | C3H | 7 days | E | TS | 103(2) | *148, 232(12) |
| CBM | 30 Lung | C57 | 5 days | E | S | 149(3) | 155(4) |
| | Heart | | | E | TS | 149(7) | 96(6, 7) |
| CBM | 32 Lung | C57 | 5 days | E | T | 67(4) | 118(4) |
| COM | 2 Kidney | C57 | 28 mths | T | S | 175(3) | 217, 264, 386(19) |
| COM | 3 Kidney | C3H | 30 mths | T | S | 106(4) | 134, 141, 159, 160, 221, 277, 407, 434 (47) |
| | Lung | | | E | S | 105(4) | 126, 160, 173, 235 (17) |
| COM | 4 Prostate | C57 | 34 mths | E | S | 196(4) | 201, 243, 283(13) |
| COM | 5 Liver | C57 | 28 mths | T | S | 75(2) | 90, 97, 152, 181, 230, 281, 355, 382, 434(47) |
| | Peritoneum | | | E | S | 117(5) | 140, 181, 230, 350(33) |
| | Kidney | | | T | S | 62(2) | 97, 98, 181, 309, 391, 434(38) |
| COM | 6 Muscle | C3H | 29 mths | E | S | — | 117, 208, 393(18) |
| | Peritoneum | | | E | S | — | 62, 146, 208, 315, 409(30) |
| | Spleen | | | E | S | 140(7) | 117, 178, 218, 310, 356(32) |
| | Testis | | | E | S | — | 315, 523(22) |

Figures in parenthesis are transfer generations.
E = Explant.
T = Trypsin.
S = Syringing.
V = Versene.
* = Eye transplant.

Tumours developed from only three of the 14 cell lines inoculated intraperitoneally. These two lines had already given rise to tumours from subcutaneous transplants at an earlier transfer generation. Details are given in Table 4.

## DISCUSSION

The results of these experiments confirm that cell lines can be readily established from mouse tissue and that this process is separate from the development of neoplastic transformation. The process of neoplastic change *in vitro* is apparently random under the conditions of these experiments except for the time of onset, although the number of experiments is too small and the range of variation too great to allow an accurate statistical analysis. The subject of transformation *in vitro* has been reviewed by Sanford [9, 10]. Only a few special points of interest will be discussed.

### *The effects of age on cell growth*

Tissue culture cell lines from almost every

*Table 3. Tumour development*

| Expt. no. tissue | Mouse strain age | Days in culture | Transfer generation | Tumour latent period (days) |
|---|---|---|---|---|
| CBM 18N Kidney | C3H 3 days | 326(5) | 27 | 102 |
| | | 246(19) | 28 | 215(eye) |
| | | 271(42) | 29 | 99 |
| | | 285(8) | 35 | 56 |
| | | 285(8) | 35 | 68 |
| | | 384(7) | 41 | 79 |
| | | 349(9) | 44 | 55 |
| CBM 31 Bladder | C3H 5 days | 205(85) | 3 | 97 |
| CBM 17 Kidney | C57 3 days | 283(14) | 23 | 63 |
| | | 350(15) | 31 | 86 |
| | | 377(10) | 41 | 47 |
| | | 397(8) | 45 | 89 |
| | | 738(8) | 63 | No tumour |
| | | 875(8) | 68 | 51 |
| | | 994(7) | 85 | 69 |
| CBM 19 Lung | C57 3 days | 521(8) | 58 | 292 |
| | | 634(10) | 73 | 152 |
| | | 728(10) | 83 | 251 |
| | | 754(7) | 87 | 187 |
| | | 776(7) | 90 | 166 |
| | | 847(7) | 100 | 160 |
| CBM 25 Kidney | C57 3 days | 286(16) | 22 | 78 |
| CBM 26 Kidney | C57 3 days | 204(15) | 12 | 152 |
| | | 253(15) | 19 | 133 |
| COM 3 Prostate | C3H 30 mths | 431(13) | 30 | 179 |
| COM 6 Brain | C3H 29 mths | 195(15) | 4 | 250 |
| | | 253(15) | 4 | 279 |
| COM 6 Nerve | C3H 29 mths | 274(10) | 11 | 18 |
| | | 358(20) | 17 | 117 |
| | | 529(10) | 37 | 83 |
| COM 6 Spinal cord | C3H 29 mths | 208(21) | 11 | 162 |
| | | 274(15) | 18 | 73 |
| COM 6 Tongue | C3H 29 mths | 334(12) | 18 | 159 |
| | | 442(7) | 31 | 103 |
| COM 1 Heart | C57 29 mths | 638(7) | 58 | 147 |
| COM 2 Lung | C57 28 mths | 229(21) | 9 | 58 |
| | | 229(21) | 9 | 63 |
| | | 280(16) | 17 | 61 |
| | | 280(16) | 17 | 60 |
| | | 320(20) | 21 | 56 |
| | | 358(17) | 26 | 68 |
| COM 4 Kidney | C57 34 mths | 190(7) | 15 | 93 |
| | | 190(7) | 15 | 135 |
| COM 4 Bladder | C57 34 mths | 387(30) | 23 | 93 |
| | | 429(13) | 28 | 316 |
| COM 5 Tongue | C57 28 mths | 243(20) | 13 | 45 |
| | | 308(8) | 22 | 39 |
| | | 338(7) | 27 | 52 |
| | | 338(7) | 27 | 54 |
| | | 440(8) | 38 | 28 |
| | | 592(7) | 71 | 15 |

Figures in parenthesis are days since last transfer.

*Table 4. Tumour development: intraperitoneal inoculation*

| Expt no. | tissue | Mouse strain | age | Days in culture | Transfer generation | Days since inoculation No tumour | Tumour development (days) |
|---|---|---|---|---|---|---|---|
| COM 5 | Kidney | C57 | 28 mths | 97 | 6 | 212 (dead)<br>240 (dead) | |
| | Liver | | | 97 | 5 | 393 (dead)<br>410 (dead) | |
| CBM 19 | Lung | C57 | 3 days | 402 | 41 | 29 (dead)<br>135 (dead)<br>440 (dead)<br>590 (dead) | |
| CBM 17 | Kidney | C57 | 3 days | 397 | 45 | | 71 (dead)<br>89 (dead) |
| COM 2 | Lung | C57 | 28 days | 174 | 2 | 369 (dead) | 582 (dead) |
| *CBM 33 | Calf | C57 | 3 days | 33 | 1 | 652 (alive)<br>652 (alive) | |
| CBM 33 | Horse | C57 | 3 days | 33 | 1 | 652 (alive)<br>652 (alive) | |
| COM 4 | Kidney | C57 | 34 days | 135 | 6 | 652 (alive)<br>652 (alive) | |
| COM 3 | Kidney | C3H | 30 mths | 160 | 12 | 571 (dead)<br>350 (dead)<br>652 (alive) | |
| | Kidney | | | 159 | 2 | 652 (alive)<br>652 (alive) | |
| CBM 18N | Kidney | C3H | 3 days | 285 | 35 | | 56 (dead)<br>68 (dead) |
| | | | | | | 159 (dead) | |
| COM 3 | Lung | C3H | 30 mths | 160 | 10 | 68 (dead)<br>652 (alive) | |
| COM 6 | Perito-neum | C3H | 29 mths | 62 | 3 | 514 (dead)<br>577 (dead) | |
| COM 6 | Spinal cord | C3H | 29 mths | 62 | 2 | 85 (dead)<br>652 (alive) | |

* Primary cultures.

organ of mice can be established without great difficulty even from tissues from very old animals. The slow growth phase appeared to be slightly longer in older animals, in C3H as compared to C57 mice, and in tumour producing lines as compared to non-tumour lines, but the range of variation is such that there are no significant statistical differences between the groups.

The growth rate of the tumour and non-tumour lines after the slow growth phase, as measured by the length of time to form a complete monolayer in the culture vessels (approximately 7 days), did not seem to differ significantly, but these are now being measured more accurately. Sawicki *et al.* [11] have reported similar findings.

The age of the donor animal does not seem to be directly correlated with tumour production since cells derived from old mice (10 of 21 lines) gave rise to tumours no more frequently than those from young animals (six of 14 lines).

*Factors influencing the development of neoplasia*

There are no reliable methods available for determining the neoplastic potential of cells *in vitro* [12, 13]. Growth of the cells after transplantation into syngeneic hosts is probably the most reliable method now available [13, 14] although it does not take into account factors such as the effects of antigenic change in the cells, cell numbers, etc. However, the range of variation in the time of onset of neoplastic change in our experiments is probably too great to be explained by the relative crudity of the test system. The tumour-producing lines can be divided into two groups. Ten of these lines had almost certainly undergone malignant

change before 200–250 days in culture (this includes two lines which were not tested until after 283 and 286 days).

The time taken is of the same order as that found by other workers (e.g. [9, 10, 11]) although longer than that taken for embryonic tissues [15]. The actual time seems to be more important than the number of transfer generations which ranged from three to 58 in our series. This would suggest that the total number of cell divisions in the whole population may not be of significance, particularly as the slow growth phase is slightly (but not significantly) longer in the tumour lines. One cell line from a young animal and one from an old animal gave rise to tumours before the end of the slow growth phase. The second group of tumour lines, all of which had been adequately tested (Table 1), did not produce tumours until 334 to 638 days *in vitro*. Five of these were from old animals. The significance of this finding is not clear since five of the lines in the first group were also derived from old tissues.

Sanford [9], in her review, concluded that spontaneous neoplastic transformation is a reproducible phenomenon with a predictable time of occurrence in all cultures in a given system. Our results differ from those of Evans, Sanford and their colleagues (reviewed in [9, 10]) in that many of our cell lines, although easily maintained in culture, have not undergone neoplastic change even though they have been maintained *in vitro* for a considerable time. Some of these may yet become neoplastic the longer they are kept in culture. This difference may be due to differences in the mouse strains, the age of the tissues from which the cultures have been prepared, differences in culture conditions, e.g. interval between medium changing or to differences in the serum supplement used (e.g. [16, 17]). However, none of these factors can explain the variation in the results obtained in our laboratory using similar methods for all the cultures. A number of other non-neoplastic mouse cell lines have been reported by other workers (see [10]). Antigenic changes are known to occur in cells in culture (e.g. [18, 19]) and it is possible that the failure of some of our lines to grow after mouse implantation may reflect antigenic changes in the cells rather than the absence of neoplastic transformation.

Our results also differ from those of McCarthy [19] who found that tumours developed from cell cultures maintained *in vitro* for brief periods (34–85 days) if the cells were inoculated intraperitoneally. There is no obvious explanation for this discrepancy.

Aaronson and Todaro [20] have reported that cells grown under conditions in which cell concentrations and the number of cell contacts were high underwent neoplastic change whereas those in which cell contacts were reduced or prevented did not. In our experiments, however, both tumour and non-tumour lines were maintained under conditions of high cell density and contact.

Barski and Cassingena [21] suggested that trypsinisation might affect the development of neoplastic change but our experiments using trypsinised and non-trypsinised cells for the establishment and subsequent transfers of cell lines show that this relationship is not invariable.

The relationship between chromosome number and structure and neoplastic change *in vitro* has been investigated by many workers (e.g. [17, 22]). The relationship of chromosome abnormalities to the establishment of cell lines and to neoplastic change is being examined using our cell lines.

Our experiments have so far thrown little light on the non-specific tumour incidence. This is hardly surprising since a study of the ultrastructure of the tissue-culture cells, described in the accompanying paper [3] has shown that under the conditions of our experiments only two main cell types, probably derived from the vascular system, have been maintained in culture, whatever the organ of origin. The parenchymal cells do not appear to survive.

There is no evidence from these experiments to show whether selection or alteration in cell character of those cells which do survive is responsible for the development of neoplastic change. Cloning experiments from early cultures to investigate this are in progress. Once neoplastic change has developed, the degree of malignancy of each cell line, as measured by the length of time from inoculation of cells to the development of a visible tumour, remains more or less constant (Table 3). The relationship of these "laboratory tumours" to spontaneous tumours is uncertain but they may provide a useful system for the study of changes during the development of neoplasia.

## SUMMARY

*Thirty-six cell lines were established from various organs of embryo* (18 *days*), *young*

*(3–20 days) and old (28–34 months) C3H and C57BL($a^t$) Icrf mice. Six of 14 lines from young mice and 10 of 21 lines from old mice produced tumours on subcutaneous inoculation into syngeneic mice after 190–638 days in culture (3–58 transfer generations). Neoplastic transformation did not seem to be related to the length of the slow growth phase before rapid* in vitro *growth began, final growth rate, method of initiation or transfer of cultures, extensive cell contacts, or age or strain of the donor tissue.*

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# Lymphocyte-Mobilizing Agents: Effects of Polymethacrylic Acid on Transplantable Lymphoma in Rodents*

S. ORMAI,† A. HAGENBEEK, and D. W. VAN BEKKUM
*Radiobiological Institute TNO, Rijswijk (ZH)*
*and Department of Radiobiology, Medical Faculty Rotterdam, The Netherlands*

## INTRODUCTION

A NUMBER of different polyanions, among them heparin [1] and various synthetic polysaccharide sulphates [2], have been reported to induce lymphocytosis in rats. On the basis of histological changes occurring in the lymphatic tissues following administration of these substances, it was concluded by Sasaki *et al.* [3] that the lymphocytosis was due to a mobilization of cells from the lymph nodes and the spleen.

In 1969 Ormai and De Clercq [4] found that polymethacrylic acid (PMAA) is exceptionally effective in this respect and furthermore the authors provided additional evidence for the lymphocyte-mobilizing (LM) activity of compounds of this type, from the results of lymphocyte counts in the thoracic duct as well as in the peripheral blood of rats.

LM activity is of interest in view of its possible application as an adjuvant in the treatment of leukaemia with extracorporeal irradiation of the blood (ECIB) and in other applications of ECIB such as immunosuppression. In the case of leukaemia, it is obviously required that the LM agents also act on malignant cells and, since information of this kind was not available in the literature, we have investigated the effects of PMAA in two animal models: a transplantable mouse lymphosarcoma and a transplantable lymphoma in the rat. Both tumours grow preferentially in the central lymphatic organs and peripherization occurs only in the very last stages of the disease, which seems to fit the objectives presented by the clinical situation.

In both systems, PMAA was found to exhibit strong tumour cell mobilizing effects, the details of which will be presented.

## MATERIAL AND METHODS

*Mouse leukaemia*

The tumour employed was classified as lymphosarcoma 2028, that arose spontaneously in a C57BL/Rij female mouse and was carried through successive passages in C57BL mice in this Institute. Cells from passages 206–225 were used in the present studies, and were routinely collected from the greatly enlarged spleens of animals in the last stages of the disease following intravenous inoculation with tumour cells. For the experiments 14–17-week old C57BL females weighing between 20 and 28 g, were inoculated i.v. with $10^6$ cells from a leukaemic spleen-cell suspension.

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*Requests for reprints should be sent to D. W. van Bekkum, Radiobiological Institute TNO, Rijswijk (ZH), The Netherlands.
†Fellow of the Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary during 1968, and visiting fellow of the Organization for Pure Scientific Research (ZWO) in The Netherlands during 1969.

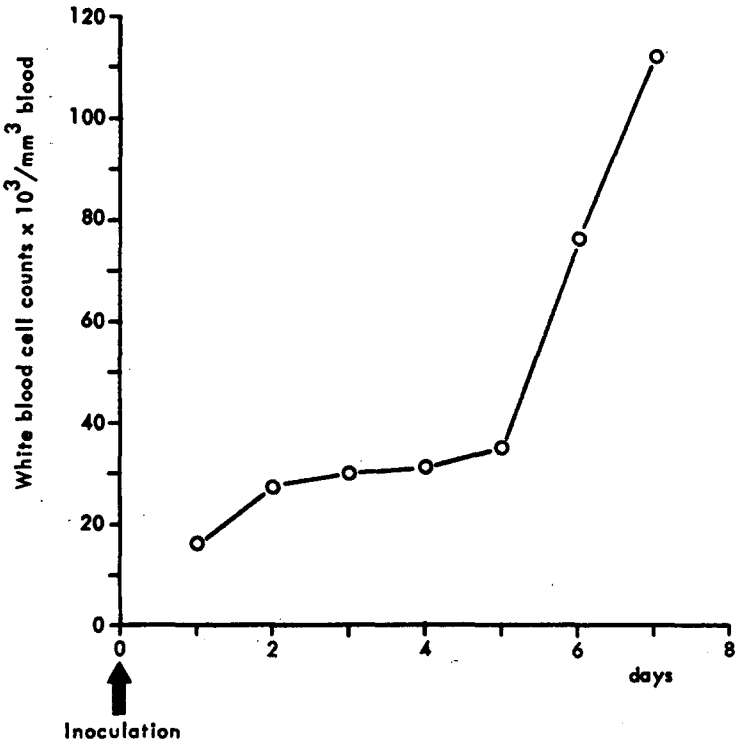


*Fig 1. Peripheral blood WBC counts in WAG/Rij rats after inoculation of Shay leukaemia cells.*

Normally, very few tumour cells appear in the peripheral blood, even in the terminal stages.

*Rat leukaemia*

The Shay chloroma was obtained from Dr. W. C. Moloney, Harvard Medical School, Boston, in 1968, as a subcutaneously growing tumour and transferred as a cell suspension to inbred WAG/Rij rats, in which it has been transferred by the s.c. route for 13 passages; subsequent passages were made i.v. by using peripheral blood from rats that showed high number of leukaemic cells in the peripheral blood. For the experiments ±8-wk old male WAG rats weighing 140–160 g received $10^7$ peripheral tumour cells i.v. Following inoculation the white-blood cell counts of the peripheral blood rise slightly during the first 5 days, with only few malignant blast cells present. After the 5th day a steep increase of tumour cells in the blood occurs (Fig. 1) and the animals die on the average on the 7th day. At autopsy the lymphatic organs and the liver are uniformly greatly enlarged with tumour growth.

*Polymethacrylic acid*

PMAA was kindly provided by Prof. P. de Somer, Rega Institute, Louvain, where it had been synthesized by polymerization of metacrylic acid in the presence of benzylperoxide [5]. The product had a molecular weight ranging between 25 000 and 1 175 000 and was administered i.v. as a polydisperse solution in phosphate buffered saline (PBS, pH 7·2). Control animals received PBS only.

*Blood cell counts*

Samples were taken from the tail by cutting the veins and total white blood cell counts were made with a Bürker type haemocytometer in Türk's solution. To determine the proportion of leukaemic blast cells, differentiation of May–Grünwald–Giemsa stained blood smears from the same samples was performed.

## RESULTS

The acute effects of PMAA administration on the lymphosarcoma-bearing mice was studied by injecting groups of mice with 40 mg of PMAA/kg body weight i.v. on the 7th day following inoculation of $10^6$ cells from the spleen of lymphomatous mice. Just before and 4 hr after the PMAA administration, blood samples were taken. Accurate differentiation between tumour cells and lymphocytes proved extremely difficult, therefore total mononuclear cell counts and polymorphonuclear counts were recorded instead. The data in Table 1 show that PMAA caused nearly a doubling of the mononuclear cell content of the peripheral blood. This increase was statistically significant and must have been due, at least partly, to an increased number of lymphoma cells, since these cells already constituted the majority of the mononuclear cells before the

*Table* 1. *Effect of a single injection of PMAA on mono- and polymorphonuclear white-blood cell counts of leukaemic C57BL mice*

| Treatment | | Before | 4 hr after |
|---|---|---|---|
| | | Cell No. per mm³ (×10³) | |
| PBS | Mononuclear | 33·1 ±2·1 | 37·7 ±6·0 |
| $n=7$ | Poly. | 23·5 ±2·6 | 37·5 ±8·9 |
| PMAA | Mononuclear | 32·5*±2·6 | 62·8*±7·5 |
| $n=6$ | Poly. | 33·0 ±6·6 | 52·6 ±8·1 |

Values represent means±S.E.
*Difference between these values is statistically significant ($P<0·02$).
All mice were inoculated with $10^6$ lymphoma cells 7 days prior to the test.
Dose of PMAA: 40 mg/kg body weight i.v.

*Table* 2. *Effect of* 3 *daily injections of PMAA immediately following tumour-cell inoculation of mice on peripheral blood cell counts taken on the 7th day*

| Treatment | | Cell No. per mm³ (×10³) | |
|---|---|---|---|
| PBS | Total white blood cells | 37·0±7·5 | $n=7$ |
| PMAA | Total white blood cells | 63·5±5·3 | $n=8$ |

Values represent means±S.E.
Difference between total cell counts in the two groups is statistically significant ($P<0·02$).

injection of PMAA. The polymorphonuclear cells also rose, but a similar rise was noted following the injection of PBS and it can therefore not be attributed to the PMAA.

Two other groups of mice were similarly inoculated with $10^6$ spleen cells from lymphosarcomatous mice. One group received 20 mg of PMAA per kg body weight on each of three subsequent days thereafter; the other group served as controls and were injected with PBS on the same days. On the 7th day after tumour-cell inoculation peripheral blood cell counts and smears were made. In the PMAA-treated mice, total white cells were significantly higher than in the PBS-treated controls (Table 2).

Inspection of the smears showed that the majority of the cells in both groups were lymphoma cells, so that the higher counts in the PMAA-treated group of mice was at least partly due to an increased number of tumour cells. It was also noted in mice that were sacrificed at that time, that PMAA treated animals generally had larger spleens than the PBS controls. This experiment has been repeated several times with slight variations and essentially similar results were obtained each time.

An acute mobilization experiment was performed with rats on the 5th day after i.v. injection of $10^7$ peripheral Shay lymphoma

*Table* 3. *Effect of a single dose of PMAA on peripheral blood cell counts of rats with Shay chloroleukaemia*

| Treatment | | Cell No. per mm³ (×10³) | |
|---|---|---|---|
| | Mononuclear | Before | 3 hours after PMAA |
| PBS | total | 20·5 | 25·4 |
| $n=4$ | Ly. | 14·9 | 20·4 |
| | Bl. | 5·6 | 5·0 |
| | Poly. | 10·1 | 12·4 |
| | Mononuclear | | |
| PMAA | total | 22·2±1·5* | 52·9±1·8 |
| $n=5$ | Ly. | 16·4±1·5† | 36·3±1·9 |
| | Bl. | 5·8±0·5‡ | 16·6±2·1 |
| | Poly. | 8·2±0·9§ | 14·3±3·2 |

Values represent means±S.E.
All rats were inoculated with $10^7$ peripheral tumour cells 5 days prior to the test.
Ly: cells identifiable as lymphocytes and non-identifiable cells.
Bl: cells identifiable as tumour cells.
Statistical evaluation of differences: *$P<0·001$, †$P<0·05$, ‡$P<0·01$, §$P<0·5$.

cells. At that time the tumour-cell content of the peripheral blood is still relatively small. Just prior to and 3 hr after i.v. injection of 40 mg of PMAA per kg body weight blood samples were taken and analyzed for lymphocytes, lymphoma blast cells, and polymorphonuclear cells. Treatment with PMAA resulted in a sharp increase of both types of mononuclear cells as is shown in Table 3. It was frequently difficult to differentiate tumour blast cells from lymphocytes, in which cases the cells were listed as lymphocytes. The true proportion of blast cells is therefore likely to be higher than indicated by the values in Table 3.

Only a relatively small increase of granulocytes was observed in the PMAA-treated animals.

## DISCUSSION

The results presented here leave no doubt that PMAA has the property of mobilizing both normal and malignant cells of the lymphoid series. Whether the quantity of leukaemic cells that is being mobilized is sufficiently large to be of practical value in increasing the effects of ECIB remains to be established. A suitable leukaemia model for the study of ECIB in rats has not yet been developed and moreover the technique of extracorporeal irradiation itself is difficult to perform in small animals. In preliminary experiments with larger animals, such as dogs and M. rhesus monkeys, PMAA has also been found to mobilize normal lymphocytes, but leukaemic animals of these species are not readily available.

At the moment, it does not seem justified to start investigations of PMAA in leukaemic patients for at least two reasons. Firstly, relatively little information is available about the toxicity of PMAA. Secondly, in the mouse strong indications were obtained that PMAA administration accelerates the growth of the leukaemic cell population. If this can be confirmed, its quantitative significance relative to the beneficial effects of killing mobilized cells by ECIB will have to be determined, before the clinical use of PMAA can be initiated.

Apart from the obvious practical implications of chemical mobilization of normal and malignant lymphoid cells, the mechanism of action of LM agents remains to be investigated.

**Acknowledgement**—The authors' thanks are due to Miss E. M. Schotman and Miss M. G. C. Platenburg for expert technical assistance and to Prof. P. de Somer, Rega Institute, Louvain, Belgium, for providing the polymethacrylic acid.

## SUMMARY

*The lymphocyte mobilizing agent, polymethacrylic acid (PMAA), was administered to mice carrying systematic transplantable lymphosarcoma and into rats with Shay chloroleukaemia. Significant increases in tumour cells were found in the peripheral blood several hours after PMAA injection, indicating that malignant cells of the lymphoid cell series are similarly affected by PMAA as normal cells.*

*The finding of signs indicating an acceleration of tumour growth in the PMAA-treated animals and the lack of information on PMAA toxicity preclude its clinical application as an adjuvant to extracorporeal irradiation of the blood.*

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# Cell Proliferation in Neuroblastoma*

H. P. WAGNER and H. KÄSER

*Swiss Center for Clinical Cancer Research and Department of Pediatrics, University Hospitals, Berne, Switzerland*

## INTRODUCTION

CLINICAL SITUATIONS permitting an adequate evaluation of cell proliferation in solid tumors are rare. Since information on essential parameters characterizing the division of neoplastic cells in man is desirable and since the proliferative characteristics of neuroblastoma cells have not yet been investigated *in vivo*, the results of a cytokinetic study in a patient with generalized neuroblastoma are reported.

## MATERIAL AND METHODS

*A short case history* of F.L. has been published previously [1]. This girl was first admitted at the age of 14 months because of an osteolytic lesion in the distal part of the left fibula which was subsequently found to be a metastasis of a neuroblastoma originating in the left adrenal. After a temporary response to radio- and chemotherapy, the patient developed widespread disease with multiple bone lesions. Extensive neoplastic invasion of the spinal canal resulted in a complete paralysis of both lower extremities associated with a total loss of sensation. Simultaneously, a rapidly growing lump was observed below the left knee above the proximal part of the fibula. At this time the following study was performed in accordance with standards for clinical research established at our institution.

First, 0·2 μCi/g body weight of tritiated thymidine ($^3$H-TdR: New England Nuclear. Specific activity: 6·7 Ci/mM. Concentration: 1 mCi/ml) was given i.v., in a single dose at noon. Tumor cell samples were then removed by aspiration, without anesthesia, from different parts of the metastasis below the left knee, at time intervals shown in Figs. 1 and 2. Smears were prepared immediately from each sample, air-dried, fixed in absolute methyl alcohol, processed for autoradiography with liquid emulsion (Eastman Kodak NTB–2) and exposed during 44 days in dehumidified air at 4°C. After development and fixation, the smears were stained with Giemsa solution (concentration 1:50; pH 6·0) dried and mounted with coverslips. From each sample 80 to 197 neuroblastoma cells in mitosis and 1500 to 2500 neuroblastoma cells in interphase were examined and the number of grains overlying the nuclear material determined. Interphase cells were classified according to relative nuclear size, size class 1 representing cells with the smallest, size class 6 those with the largest nuclei. Since the nuclear size was influenced by the thickness of the smears only the relative size of the nuclei in each area evaluated was assessed. Size class 1 comprised 74·8±6·4, size classes 2 and 3 42·5±5·8 and size classes 4–6 9·7±1·7% of all neuroblastoma cells in interphase. Only cells with 4 or more grains were considered to be labeled.

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## RESULTS

*Mitotic index*

During the evaluation of 22,000 neuroblastoma cells in interphase on smears from different samples a total of 126 tumor cells in mitosis was found. The overall mitotic index is 0·572%.

*Labeling index and labeling intensity of neuroblastoma cells in mitosis*

The change in the labeling index and the labeling intensity of neuroblastoma cells in mitosis, as a function of time after $^3$H-TdR injection, is shown in Fig. 1. In the mitotic labeling index curve a large initial wave but no definite second ascent was observed. In the

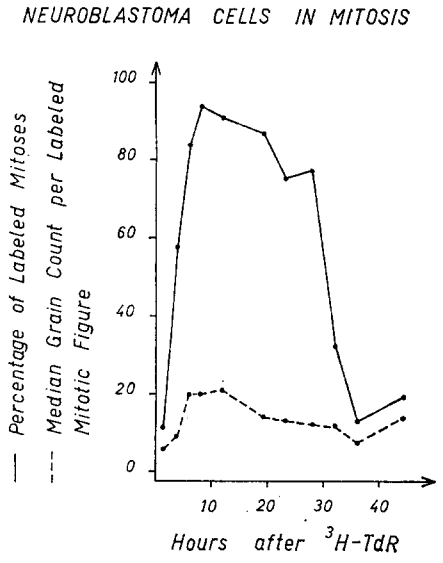


*Fig.* 1.

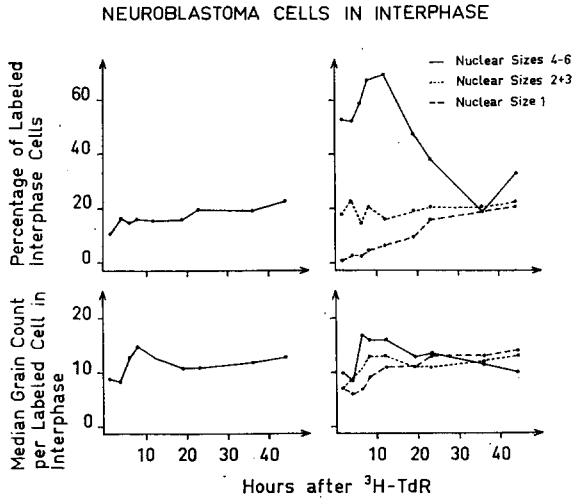


*Fig.* 2.

labeling-intensity curve a first peak at 6–12 hr and a second rise at the end of the observation period was noted.

*Labeling index and labeling intensity of neuroblastoma cells in interphase*

In Fig. 2 the labeling index and median grain-count curves are shown (i) for all neuroblastoma cells in interphase (left) and (ii) for neuroblastoma cells belonging to different nuclear-size classes (right).

One hour and a half after $^3$H-TdR administration 11% of all neuroblastoma cells and 53, 18 and 1·3% of those with large, intermediate and small nuclei respectively were labeled.

During the period of observation the relative number of labeled neuroblastoma cells increased slightly (Fig. 2, upper left) while the labeling intensity, after an initial rise, remained more or less constant (Fig. 2, lower left).

As shown in the diagram on the upper right of Fig. 2, the labeling index of cells with small nuclei (broken line) increased gradually from 1·3 to 20·6%. The labeling index of cells with large nuclei (solid line), after a transitory rise, fell until values similar to those of cells with small nuclei were reached. Little change was observed in the labeling index of cells with nuclei of intermediate size. The median grain count curves (Fig. 2, lower right) indicate that cells with large nuclei initially had higher median grain counts than those with small nuclei. After an initial rise, the median grain-count of cells with large nuclei decreased whereas that of cells with small nuclei increased.

## DISCUSSION

As shown elsewhere [2], a combined analysis of mitotic labeling index and mean or median grain count data after pulse-labeling with $^3$H-TdR necessitates the least number of assumptions if the time parameters characterizing the cell cycle of a given cell type are to be assessed *in vivo:*

(1) Adequate incorporation of the cell marker by dividing neuroblastoma cells is demonstrated by the fact that over 90% of all mitotic figures were labeled 8 hr after $^3$H-TdR administration.

(2) Uniformity of the cell population, although not investigated by chromosomal studies or cytophotometric DNA determinations in single cells, is not unlikely since morphologically only one type of mitotic figures could be observed and since all cell samples were removed from the same rapidly growing metastatic lump.

(3) A diurnal variation of the mitotic index was not found when the mitotic indices of different samples were compared.

(4) Re-utilization of labeled nuclear material, at least during the period of observation, did not play a major role since the mitotic labeling index fell to low values between 28 and 32 hr after $^3$H-TdR injection. If there is early and massive re-utilization, the labeled mitoses curve, after its first rise, remains elevated and arbitrarily set high background levels are required in order to visualize periodic changes in the mitotic labeling index [3].

From the data shown in Fig. 1 the following estimates of parameters characterizing the cell cycle of neuroblastoma cells *in vivo* can be made:

(i) minimum duration of $G_2$: approximately 1–2 hr (time interval between $^3$H-TdR injection and first appearance of labeled mitoses).
(ii) minimum duration of $G_2$+S: approximately 28 hr (time interval between $^3$H-TdR injection and the beginning of the first significant fall in the labeled mitoses curve).
(iii) mean duration of S: 27–28 hr (time interval between mid-points of the ascending and descending parts of the labeled mitoses curve).
(iv) mitotic time: 1·4 hr (calculated from mean DNA synthesis time, initial labeling index of cells in interphase and mitotic index).

Generation time cannot be assessed accurately from the mitotic labeling index data. However, the last portion of the median grain count per labeled mitotic figure curve might indicate that daughter cells of initially labeled cells start to pass through mitosis. The labeling intensity of mitotic figures will, with some provisos, rise to a first peak when initially labeled cells pass through mitosis and rise to a second, lower peak when daughter cells of initially labeled cells divide [2]. The labeling intensity data shown in Fig. 1 are therefore compatible with the assumption of a minimum cell cycle time of approximately 35–40 hr.

The labeling index and median grain count curve shown on the left of Fig. 2 are difficult to evaluate without data on an eventual in- and/or efflux into and/or out of the cell population studied. Some information was obtained by classifying neuroblastoma cells in interphase according to relative nuclear size. The labeling index curves for neuroblastoma cells with large, medium and small nuclei shown in Fig. 2 (upper right) are similar to those observed for leukemic cells of different nuclear sizes in bone marrow of children with untreated acute lymphatic leukemia [4, 5]. One may conclude, therefore, that the neuroblastoma-cell population studied consisted of at least two compartments (one of initially labeled, proliferating cells with large nuclei and one of initially unlabeled, non-proliferating cells with small nuclei) and that there was a continuous reciprocal exchange of cells between these compartments. As in acute lymphatic leukemia [6], the majority of the non-proliferating neuroblastoma cells may have left the generative compartment only temporarily and may divide again after various periods of time.

From the data available cell loss cannot be estimated. Since none of the few pyknotic nuclei observed was labeled, rapid death of dividing cells did not appear to be an important factor.

**Acknowledgements**—We thank Miss M. Troxler, Miss T. Fuhrer and Mrs. E. Werren for their expert technical assistance.

## SUMMARY

*In a patient with generalized neuroblastoma labeling index and median grain-count determinations on tumor cells in mitosis and interphase were performed autoradiographically after pulse-labeling with tritiated thymidine. The tumor-cell samples were removed from different parts of a superficially growing metastasis. The mitotic index was* 0·57%. *Labeled mitoses data indicated a minimum $G_2$ time of* 1–2 *hr and a mean DNA synthesis time of approximately* 27–28 *hr. Mitotic-time calculations yielded a value of* 1·4 *hr. The initial labeling index of all neuroblastoma cells in interphase was* 11%. *Classification of tumor cells according to relative nuclear size permitted to distinguish at least two cell compartments: one of cells with large, initially labeled and one of cells with small, initially unlabeled nuclei. The data are compatible with the assumption that there is a continuous reciprocal exchange of cells between these compartments and that the majority of the tumor cells retain the ability to divide.*

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# The Influence of Allogeneic Inhibition and Tumour Age on the Kinetics of L1210 Leukaemia *in vivo*

Cl. CHOQUET, N. CHAVAUDRE and E. P. MALAISE

*Service de Radiobiologie Clinique, Institut Gustave-Roussy, 16 bis, avenue Paul. Vaillant-Couturier, 94 Villejuif, France*

## INTRODUCTION

THE experimental leukaemia, L1210, has been used for a long time [1], and has enabled many problems of cancer chemotherapy to be tackled. The study of Skipper and his colleagues [2, 3] has considerably increased our knowledge by establishing a precise, quantitative relation between the number of leukaemic cells injected and the mean survival times of the animals; this new technique has enabled the action of anti-cancer drugs to be measured quantitatively.

The relation between the number of leukaemic cells injected and the mean survival time of the animals is sufficiently precise for the growth curve of the leukaemia to be determined. The initial part of this growth curve is exponential; the doubling time, varying with the route of injection [3, 4] is very short, with upper and lower limits of 10 and 13·2 hr respectively. However, longer cell cycle times were observed when they were measured by the percentage-labelled mitoses method, Skipper and his group [4] reported 14·5 hr and Wheeler and his collaborators [5] calculated the leukaemia had a cell cycle time of 15·8 hr in mice. It is very surprising to find a duration of cell cycle greater than the duration of the doubling time, especially for Skipper's studies, where the measurement of the tumour doubling time and the duration of the cell cycle were made under comparable conditions.

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Two hypotheses can be proposed to explain this paradox. It is known that the duration of the cell cycle of an ascitic tumour is a function of the age of the ascites [6, 7]. It is not unreasonable to suppose that an analogous phenomenon exists for L1210 leukaemia, at least when it is growing in the peritoneal cavity — a site that is generally used for kinetic studies. For technical reasons, autoradiographic studies are generally made on animals carrying a large number of leukaemic cells, that is, on leukaemias that have been growing for a relatively long time. On the other hand, quantitative studies of the growth curves are made during an earlier phase of the leukaemia. Furthermore, the majority of kinetic studies of L1210 leukaemia have been made using (DBA/2 × C57B16) $F_1$ hybrids. There have been numerous studies of the 'hybrid effect' [8, 9, 10, 11], sometimes described as 'hybrid resistance' [9], 'allogeneic inhibition' [10] or 'hybrid hyper-reactivity' [11], the nature of this phenomenon is still under discussion. Nevertheless, it seems clear that the hybrid effect usually shows up as a slowing or even an inhibition of tumour growth. It can be questioned whether the hybrid effect can have a direct influence on the cell-cycle duration of the leukaemic cells.

The present paper describes an investigation of the kinetics of L1210 leukaemia at different times in the growth of the tumour, undertaken both in isogenic DBA 2 and in (DBA/2 × C57B16) $F_1$ hybrid mice, with a view to investigating the hypotheses proposed above.

## MATERIAL AND METHODS

*L1210 leukaemia*

The L1210 leukaemia was maintained by serial transplantation in (DBA/2×C57B16) $F_1$ mice. The tumour was transplanted routinely every 7 days. A cell suspension in MacCoy's medium [12] was prepared from the ascitic fluid of 3 mice, diluted to contain $10^5$ cells in 0·5 ml. The medium was kept at 0°C during the preparation of the suspension; the manipulations never took longer than 1 hr. No serum was added to the cell suspension. $10^5$ cells were injected intraperitoneally into each animal and, as a rule, about $10^8$ cells were present in the ascites when they were killed. In the present experiments, the same technique was used, except that the cells were transplanted after growing for only 5 days.

*Animals*

Two strains of mice were used: DBA/2, the line in which the leukaemia originated, and (DBA/2×C57B16) $F_1$ hybrids. The animals were aged 2 to 4 months; both sexes were used. They were housed 10 to a cage and given a sterilized dry food, enriched with vitamins and water *ad libitum*.

*Tumour induction*

The measurement of the number of cells required to induce tumours in 50% of the animals (T.D. 50) was made using Hewitt's method [13]. Suspensions containing different concentrations of cells were prepared, the concentration of the suspensions was increased geometrically, the coefficient between two adjacent suspensions was 3; each suspension was injected into a group of 10 animals.

*Mean survival of the leukaemic animals*

The mean survival time was studied, following the injection of a number of cells that induced leukaemia in 100% of the animals (100 or more cells per animal), for each number of cells injected, the mean survival was studied on a group of 10 animals of each strain. The animals were examined twice a day, usually about 9 a.m. and about 6 p.m. The survival of each animal was estimated as the time which elapsed between the injection and the mean between the last time it was seen to be alive and when it was found to be dead. For example, when the two successive observations were made at 9 a.m. and 6 p.m. on the same day, we have estimated that the animals found dead at 6 p.m. had died, on average, at 1.30 p.m.*

*Study of the cell cycle*

The cell cycle was studied using the method of Quastler and Sherman [14]. This study was made on animals which had received, originally, 350,000 cells. After a variable delay, according to different experiments (2·5–8·0 days), 10μCi of tritiated thymidine ($^3$H-Tdr) was injected intraperitoneally. Samples of cells were taken serially for the next 20–35 hr, smeared on slides, air-dried and fixed in methyl alcohol for 10 min. The slides were coated with Ilford K2 Nuclear Emulsion and exposed for 2–8 days at a temperature of 4°C. After developing, the slides were dehydrated for half an hour in 70% alcohol, and finally stained by the Romanowski method.

The background radiation was usually very slight a mitosis was considered to be positively labelled if it gave rise to 3 or more grains. Whatever the percentage of labelled mitoses was, no fewer than 30 labelled mitoses were counted for each time interval. Two mice were used to measure the percentage labelled mitoses at each point in time.

## RESULTS

*Tumour frequency*

Figure 1 shows the frequency of tumours induced by various concentrations of cells. It can be seen that the T.D.50, which has not been calculated precisely, is less than 10 cells in the $F_1$ hybrid and DBA/2, but it cannot be confirmed from these results whether there is a significant difference between the T.D.50 in $F_1$ hybrids and the T.D.50 in the DBA/2 mice. The T.D.100 minimum in our system is less than 100 L1210 cells in both the DBA/2 and the $F_1$ hybrids.

*Survival of the leukaemic animals*

The survival time decreased as the number of

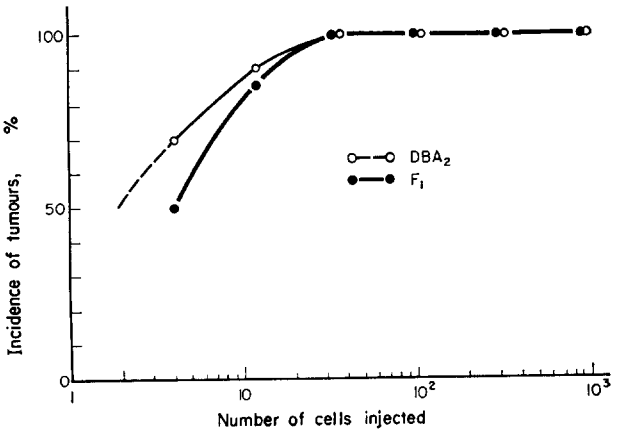


*Fig. 1. Relationship between the % incidence of tumours L1210 in DBA/2 and $F_1$ and the number of cells injected.*

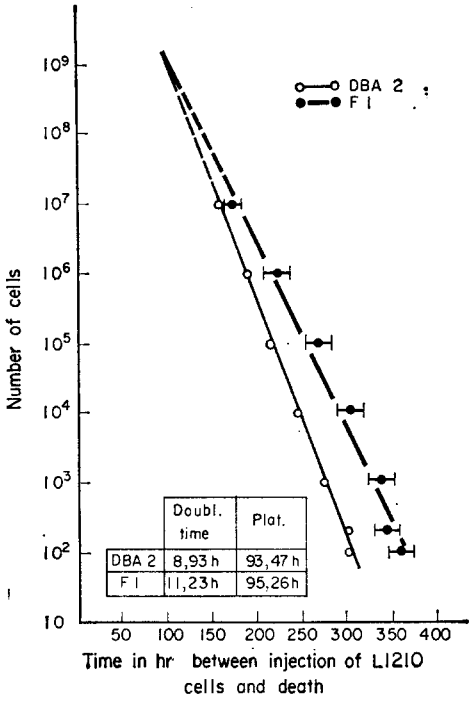


*Fig. 2. Relationship between the number of cells L1210 injected and the mean surival time of the mice (DBA/2 and $F_1$).*

leukaemic cells injected was increased; the relation between these two parameters corresponded to an exponential function for numbers of cells between $10_2$ and $10^7$ (Fig. 2). The effects of injecting more than $10^7$ cells were not investigated. There was a highly significant difference between the results obtained in the two types of animals. In DBA/2, the doubling

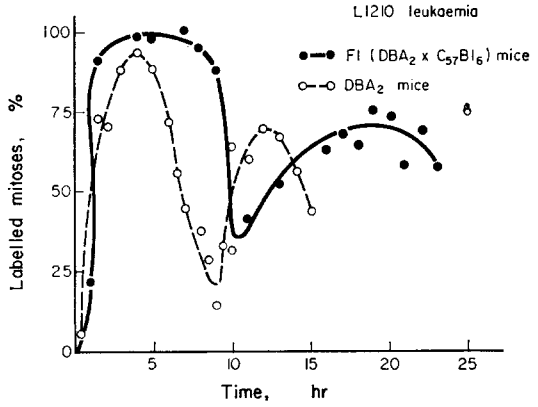


*Fig. 3. Cycle time of the L1210 leukaemic cells measured by the % of labelled mitoses (DBA/2 and $F_1$).*

time was 8·9 hr, whilst in the $F_1$ hybrid it was 11·2 hr. On the other hand, these two curves converged at a point corresponding to more than $10^9$ cells.

Extrapolation of the growth curves in Fig. 2 indicated that an injection of $1{\cdot}5\times10^9$ cells would kill DBA/2 mice in 93·4 hr and the $F_1$ hybrids in 95·2 hr.

*Cell cycle*

When measured at a time when the number of leukaemic cells in the animal was in the order of $10^7$, the cell cycle time was 9·0 hr in DBA/2 and 11·5 hr in the $F_1$ hybrid (Fig. 3). The difference between these results appears to be related to the S phase, which was longer in the hybrid animals.

*Table 1*

| | Duration of cell cycle (hr) | G2+M | S | $G_1$ | Labelled cells (%) | Growth fraction (%) | Doubling time in the exponential phase |
|---|---|---|---|---|---|---|---|
| *DBA/2 mice* L1210 — exponential phase (2·5 days after injection of $3{\cdot}5\times10^5$ cells) | 9·0 | 1·0 | 5·7 | 2·3 | 65 | 100 | 8·9 |
| *DBA/2 mice* L1210 — plateau phase (5·0 days after injection of $3{\cdot}5\times10^5$ cells) | 14·0 | 1·5 | 7·0 | 5·5 | 44 | 88 | |
| (*DBA/2*×*C57B16*) $F_1$ *mice* L1210 — exponential phase (5·0 days after injection of $3{\cdot}5\times10^5$ cells) | 11·5 | 1·0 | 8·5 | 2·0 | 57 | 78 | 11·2 |
| (*DBA/2*×*C57B16*) $F_1$ *mice* L1210 — plateau phase (8·0 days after injection of $3{\cdot}5\times10^5$ cells) | 24·5 | 2·0 | 19·0 | 3·5 | 34 | 44 | |

Calculated according to the formula:

$$\frac{\text{percentage of labelled cells}}{\text{S phase/duration of cell cycle}}$$

the growth fraction is 100% in the DBA/2 and 78% in the $F_1$ hybrid. It can be seen that the cell cycles have a duration comparable to that of the doubling time calculated from the growth curves in Fig. 2 (Table 1).

When measured 3 days before the animals' deaths, the cell cycle was prolonged in both strain of mice. It increased from 9–14 hr in DBA/2 (Fig. 4) and from 11·5–24·5 in the $F_1$ hybrid (Fig. 5). All phases of the cell cycle were more of less involved in this prolongation; similarly, there was a clear-cut fall in the growth fraction in the $F_1$ hybrids that was only slight in DBA/2.

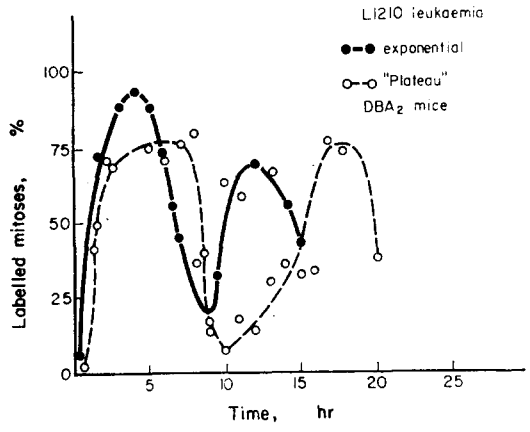


*Fig. 4. Influence of the phase of the growth curve on the cycle time of the L1210 leukaemic cells in DBA/2 mice.*

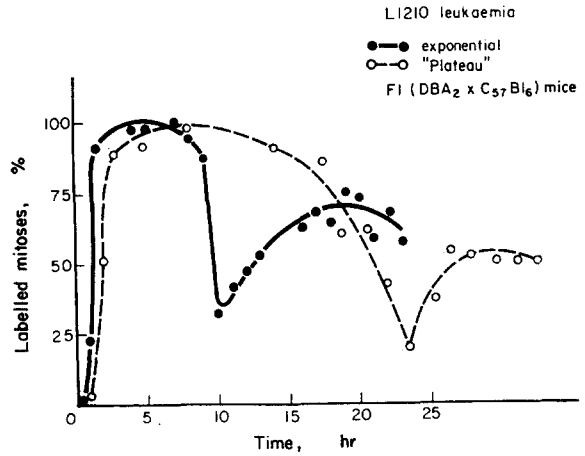


*Fig. 5. Influence of the phase of the growth curve on the cycle time of the L1210 leuakemic cells in $F_1$ mice.*

## DISCUSSION

No argument has been presented, so far, to refute that the relation discovered by Skipper between the number of leukaemic cells injected and the survival of the animal can be considered as a growth curve. On the other hand, as Skipper found, the cell cycle for L1210 had a duration greater than the cell doubling time calculated from the relation between the number of cells injected and the survival of the animals; it can be questioned if this relation corresponded closely to an initial exponential phase of the growth curve. It is hardly conceivable that a cell-cycle time could be longer than the doubling time when these two measurements are made simultaneously. In the present experiments, 2·5 days after the injection of $3{\cdot}5 \times 10^5$ leukaemic cells into DBA/2 mice, the cells had a cell cycle time of 9·0 hr and the growth fraction was 100%. If the cells divided without delay from their moment of injection, it is likely that at the time the cell cycle was studied, about $3{\cdot}5 \times 10^7$ leukaemic cells would have been present in the DBA/2 mice. The results we have obtained, using the titration method of Skipper, suggest that up to $10^7$ or fewer leukaemic cells, the multiplication seems to be exponential with a doubling time of 11·9 hr in DBA/2 mice. Overall, these results suggest that, when 100 L1210 cells are injected intraperitoneally into DBA/2 mice, these cells will undergo at least 17 divisions without any modification of the cell cycle or loss of cells.

The growth of the L1210 provides an example of exponential growth *in vivo* that can be really compared to the exponential growth which can be attained by cells growing in tissue culture [15]. However, there are technical problems in how to measure this phenomenon. Skipper's titration method is hardly able to be applied when more than $10^7$ leukaemic cells are injected; by contrast, autoradiographic studies are virtually impractical on animals containing less than $10^7$ leukaemic cells.

It can be asked if this coincidence of the duration on the cell cycle and of the doubling time in DBA/2 during the initial part of the growth curve is a unique phenomenon to L1210 leukaemia. Because of its independence of blood supply, the leukaemia is not subjected to many of the influences that inhibit the growth of solid tumours. However, this comparison cannot be taken too far, for there is very little knowledge of cell-growth rate in solid tumours when they contain less than $10^7$ cells.

In DBA/2 mice, 5 days after the injection of leukaemic cells, the cell cycle is greater than 14 hr, and simultaneously, the growth fraction

falls to 88%. This indicates between the 2nd and the 5th day, these two kinetic parameters are modified. The increased duration of the cell cycle, and the reduction of the growth fraction, lead to a reduction in the growth rate of the leukaemia, hence the age of the leukaemia has a considerable influence upon the kinetic parameters, which is in agreement with observations made on other forms of ascitic tumours [6], where, as the tumour ages, there is a progressive increase in all phases of the cell cycle.

It is unlikely, especially in DBA/2 mice, that the modification of the leukaemic-cell kinetics can be attributed to an immune phenomenon: firstly, this modification does not appear until 5 days after the injection of the cells and, secondly, it is in an isologous system.

Skipper and Wheeler both studied the cell cycle 6 days after the injection of $10^5$ cells. It is difficult to know if the age of the tumour could explain the differences between their results and ours, for the two situations are not comparable. It seems, in any case, that other factors have to be taken into account when considering Wheeler's results, for he found, both *in vivo* and *in vitro*, cell cycles with a 15·8 hr duration.

The experiments carried out simultaneously in isogeneic and $F_1$ hybrid animals, have shown that the phenomenon we have described in the isogeneic system also exists in a hybrid. During the early phase of the growth curve the duration of the cell cycle and the doubling time are comparable. Later, the cell-cycle time increases and the growth fraction is diminished.

In this later phase of growth, particularly in the hybrids, it should be noted that the growth fraction, which we have calculated from studies made on the 5th day, is reduced to 78%. The real doubling time, calculated from the formula Tc/G.F. ought in this case, to be the order of 14·7 hr, whilst the doubling time in these experiments, measured by the method of Skipper was 11·2 hr (Table 1). It is likely that the study of the cell cycle in the $F_1$ was made at a time when truly exponential growth had finished.

In general, it is known that cell loss can only be studied where the rate of cell production and the true growth curves are studied simultaneously [16]. The growth curves, measured by the Skipper's method, are impractical above $10^7$ cells. It is not possible to study the eventual loss of cells that will become apparent as the growth curve enters into a plateau. On the other hand, it can be concluded that, as far as growth in DBA/2 is concerned, the L1210 leukaemia shows no cell loss during its exponential growth phase.

These experiments have revealed another phenomenon: although the T.D.50 is low, both in the $F_1$ hybrids and in isologous mice (2–4 cells under the conditions of our experiments), it is evident that there exists what may be called an allogeneic inhibition effect. It is not possible to say whether this inhibition influences the T.D.50, but it is certain that it leads to an increase of the duration of the cell cycle and of the doubling time which, during the exponential growth phase, is 27% longer in the $F_1$ hybrid than it is in the DBA/2. The S phase seems to be particularly involved in this inhibition. Although this observation does not allow us to elucidate the mechanism of allogeneic inhibition, it shows that in our experimental system it leads solely to an increase of the tumour-cell cycle time, but does not seem — at least in the present case — to cause an increased death of the tumour cells. From a practical point of view, examinations of the results shown in Fig. 2 seem to indicate that the titration method has given more precise values in the isogeneic mice than in the hybrids. Allogeneic inhibition causes both a prolongation of the mean survival time and an increased scatter of the results.

Finally, it should be noted that the increase of cell-cycle time, which occured rapidly and could be the result of the age of the leukaemia or of allogeneic inhibition or both these factors, is also rapidly reversible. These experiments have all been made using cells coming from $F_1$ hybrid mice. At the time they were prepared for injection their cell-cycle time was 11·5 hr, and 2·5 days after their injection into the DBA/2 mice the cells had a cell-cycle time of 9 hr.

* For the experiments on the tumour frequency, the mean survival of the leukaemic animals, and the study of the cell cycle, the animals were divided several times at random. The first division occurred when the animals were put into different cages, having come from a common group; they were re-divided at the time of injection of the leukaemic cells and divided once again into groups in which they remained until the end of the experiment. The purpose of this multiple randomization was to try to prevent 'cage effects'.

**SUMMARY**

*We have confirmed that the cells of a leukaemia, grown as an ascites, undergoes a lengthening of cell-cycle time and diminishing growth fraction. In isologous mice, the initial growth*

*phase of the L1210 leukaemia is truly exponential and the duration of the cell cycle closely corresponds to the doubling time. The slow increase of cell cycle time, at least in the isologous mice, does not seem to be able to be explained as an immune phenomenon. Allogeneic inhibition has been demonstrated, both by an equivalent increase of the doubling time and the duration of the cell cycle, which occurs during the initial phases of tumour growth.*

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# DNA synthesis during the life cycle of L cells: Morphological, histochemical and biochemical investigations with arabinosylcytosine and thioarabinosylcytosine*

VAGN BREMERSKOV,† PETER KADEN‡ and CHRISTIAN MITTERMAYER‡

†*The Department of Pathology, Finsen Institute, Copenhagen, Denmark*
‡*The Department of Pathology, Medical School, University of Freiburg, Germany*

## INTRODUCTION

SINCE the synthesis of the abnormal cytidine derivative 1-beta-D-arabinofuranosylcytosine (ARA-C) by Hunter [1] and by Walwich *et al.* [2], many investigators have found this compound to have interesting properties in human [3, 4] and experimental neoplasia [5–9] both *in vivo* and *in vitro*. ARA-C inhibits the growth of certain transplantable and established rodent tumours [6, 9] and leukemias [7, 10], murine lymphoblast cells in culture [10–12], DNA synthesis in vira [13–15], bacteria, animal cells [8, 13, 16–20] and whole organisms [21, 22] and in more malignant and normal cell lines in culture [3, 19, 23–25]. The cytostatic activity of ARA-C against vaccinia virus [15], murine lymphoblasts [10], L-mouse fibroblasts [25] mouse tumours [7] and regenerating bone marrow [24] is reversed to a varying degree by deoxycytidine. In studies on purified enzymes from *E. coli* it was observed [23] that DNA-dependent-RNA-polymerase and polynucleotide phosphorylase did not effectively catalyse the incorporation of ARA-CDP or ARA-CTP into RNA and that DNA-polymerase did not incorporate ARA-CDP into DNA. Silagi [25] found a measurable amount of ARA-C incorporated into DNA of L cells and that the RNA and protein synthesis continued in spite of some incorporation into RNA. On the other hand, it has been found, that ARA-C inhibits the incorporation of $^{3}$H-UdR into DNA (via the *de novo* synthetic pathway of TTP) but not into RNA [10–12]. Based on the effect of deoxycytidine it has been postulated [10] that ARA-C acts to block the reduction of CDP to dCDP. This explanation has been supported by the finding, that ARA-C enhances the incorporation of deoxycytidine into

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Abbreviations: ARA-C: 1-beta-D-arabinofuranosylcytosine HCl; Thio-ARA-C: Thio-1-beta-D-arabinofuranosylcytosine HCl; RNA: Ribonucleic acid; DNA: Deoxyribonucleic acid; dCMP-deaminase: Deoxycytidine-monophosphate-deaminase; CdR: Deoxycytidine; ARA-CDP: ARA-C-diphosphate; ARA-CTP: ARA-C-triphosphate; CDP: Cytidine-diphosphate; dCDP: Deoxycytidine-diphosphate; 2-$^{14}$C-dCMP: Deoxycytidine-5′-monophosphate-2-$^{14}$C; 2-$^{14}$C-Thd: Thymidine-2$^{14}$C; dCTP: Deoxycytidine-triphosphate; $^{3}$H-UdR: Uridine-(U)-$^{3}$H; PCA: Perchloric acid; PPO: Toluene-2,5-diphenyloxazole; POPOP: 1.4-di-2-(5-phenyloxazolyl) benzene.

DNA through a stimulation of deoxycytidine-kinase which highly favours the phosphorylation of deoxycytidine over that of ARA-C [11, 18, 20]. Further defence mechanisms have been observed by Kit *et al.* [8] who found that treatment of green monkey kidney cells with ARA-C, resulted in a 4–7 times increase in thymidine-kinase activity. Using the CV-1 strain of these cells they also found [8] an increase in dCMP-deaminase activity. In cells resistant to ARA-C through continous low-dose treatment an increased poolsize of dCTP has been found [12] which would be consistent with an increased activity of some CDP-reductase.

The knowledge we have about the functions of ARA-C in normal and neoplastic cells and tissues and the mechanisms by which the cells protect themselves from the lethal effects of the drug has been obtained in part from quite different systems. We therefore found it of interest to compare some of the effects found in vira, bacteria and more or less complicated mammalian cell systems quantitatively with those found in L cells. It was our hope that such comparisons might enable us to construct a common theory for the qualitative function of ARA-C in cellular metabolism.

## MATERIAL

2-$^{14}$C-dCMP was purchased from New England Nuclear Corporation, Boston, Mass. $^{14}$C-Thd and 1-$^{3}$H-leucine were obtained from the Radiochemical Centre, Amersham, England. dCMP, dUMP, dCTP, Thd, TMP and TTP were obtained through California Corporation for Biochemical Research, Los Angeles, Cal. Thio-ARA-C and ARA-C were generous gifts from Dr. Charles W. Mushett, Merck, Sharpe & Dohme, Research Laboratories, Rahway, N.Y.

The rest of the chemicals used in these experiments were analytical grade. Whatman DE 81 diethylaminoethyl cellulose paper (0, 4 mEq/g) was purchased from Serva Entwicklungslabor, Germany.

## METHODS

(1) *Cell line*

Throughout the investigation a line of L cells were used and propagated in a modified Eagle medium as described previously [26]. Synchronization of cell division was accomplished by mechanical selection of cells in mitosis. The synchronous system was characterized before [26, 27].

(2) *Estimation of the rate of DNA and RNA synthesis*

DNA and RNA synthesis were assayed by growing the cells in medium containing 10 μg thio-ARA-C or ARA-C per ml medium. After 1, 3, 6, 9 and 12 hr cells in one bottle containing thio-ARA-C and in one containing ARA-C treated cells were pulselabelled with $^{14}$C-Thd (0·5 μCi/ml, 5 mCi/mM) and with $^{3}$H-UR (1 μCi/ml, 2·5 mCi/mM), after removal of the antibiotic containing medium, for 15 min at 37°C. The reaction was stopped by washing 3 times with 5 ml ice-cold 0·27 M PCA. The acid precipitate was hydrolysed by adding 5 ml 0·5 M NaOH. Of the hydrolysate, aliquots were taken for estimation of the $^{14}$C-Thd and $^{3}$H-UR incorporation into the acid insoluble fraction.

This was done by mixing, in a screw capped bottle, 1 ml of the hydrolysate with 15 ml of Bray's scintillation fluid, consisting of: PPO 8 g, POPOP 40 mg, ethylene glycol 20 ml, methanol 100 ml and naphthalin 120 g per l of dioxan. Counting was performed in a Beckman 200 scintillationspectrometer. Cells grown without addition of antibiotics were treated in the same way.

(3) *Determination of bulk DNA, RNA and proteins*

This was done according to Burton [28], Ceriotti [29] and Lowry [30] respectively.

(4) *Assay of dCMP deaminase*

dCMP-deaminase activity was measured by a modification of an isotopic method described previously [31, 32]. Each reaction tube contained in a final volume of 1 ml the following substances at the indicated concentrations: $3{\cdot}3\times10^{-2}$ M $Na_2HPO_4$–$KH_2PO_4$ buffer, pH 7·3, $2\times10^{-4}$ M dCTP, $3\times10^{-2}$ M NaF, $2\times10^{-4}$ M $MgCl_2$ and enzyme extracts containing about 125–500 μg protein.

Crude enzyme extracts were prepared by sonication. This was done by sonicating the cells 16 times for 0·1 sec (Branson sonifier) with the power supply tuned to 8 d.c. A. For each 4 sonications (of the 16) the test tube was cooled in an ice bath for approximately 10 sec. In experiments with asynchronous cultures 1 to $3\times10^{6}$ cells were sonicated while it was done on $2{,}5\times10^{5}$ cells in synchronous culture experiments. In all experiments sonication was done in 1 ml of the above solution.

The efficiency of this procedure was continuously controlled in the light microscope.

Since the soluble fraction is known to contain all the enzyme activity [33], centrifuga-

tion of the sonicates were carried out at 30 000 *g* for 1 hr at 0°C. After centrifugation, the samples were prepared in the following way: 0·85 ml of the supernatant was mixed with 0·15 ml of three times concentrated buffer solution (as above) to which was added 2-$^{14}$C-dCMP (1 μCi/ml, 0·1 mCi/mM). Incubation was carried out at 37°C in a water-bath for 60 min. The reaction was terminated by boiling for 3 min. After recooling, 0·2 mg of each of the two carriers dUMP and dCMP were added.

Separation of the nucleotides was done by descending paper chromatography on DEAE strips (1·5×36 cm²) using a solvent system containing equal parts of 4 M formic acid and 0·1 M ammonium formate in a saturated atmosphere. Initially the separation was controlled in u.v. light. Later this step was omitted because the method gave highly reproducible results. Usually, chromatography was carried out for about 5 hr. $R_f$ values for dCMP, dUMP and TMP respectively were 0·95, 0·5 and 0·08. In the experiments with asynchronous cultures, a small TMP-synthetase activity was observed, which varied linearly with the dCMP-deaminase activity. However, in the synchronous cultures no synthetase activity could be detected, probably because less cellular material was used. After the separation the strips were dried on cheesecloth, cut in 1·5 cm² squares and put into screw capped bottles containing 5 ml Bray scintillation fluid. The samples were counted for 20 min in a Beckman 200 scintillation counter. The enzyme reaction was linear with respect to time and protein concentration.

(5) *Assay for DNA-polymerase activity*

This method has been described in detail elsewhere [34].

(6) *Morphological and quantitative cytophotometric assays*

Tissue cultures for morphological and quantitative cytophotometric assays were prepared in the following way. After introduction of coverslips (12×64 mm) into tissue-cultures bottles (Roux type, Schott und Gen, Mainz) these were sterilized by heating and recooled to 37°C. Cells from a cell suspension were added and allowed to grow in normal medium for 2 hr. After this period new medium was supplied, prewarmed to 37°C and enriched by addition of either thio-ARA-C (10 μg/ml) of ARA-C (10 μg/ml) representing a final concentration of approximately $4\times10^{-5}$ M. The control cultures were grown without addition of antibiotics after replacement of the medium. At different hours, coverslips from one thio-ARA-C and from one ARA-C bottle were removed and fixed in a modified Kahle's fixative (95% ethylalcohol–formalin–glacial acetic acid) (15 : 6 : 1) for 45 min at 0°C.

Then the coverslips were transferred to 70% ethylalcohol. From each bottle one coverslip was passed in an alcoholic series and stained according to Feulgen [40, 41] after hydrolysis for 12 min in 1 N HCL at 60°C. From thio-ARA-C treated cultures another coverslip was stained with chrome-alum-gallo-cyanin-coriphosphin (CAGC) [35, 36]. This method is based on the staining of DNA and RNA according to Einarson [37] followed by hydrolysis in 1 N HCL at 60°C for 12 min and staining of the free aldehyde-groups by coriphosphin [29]. A third coverslip from each bottle was stained with naphthol Yellow S, according to Deitch [38]. The relative absorption of nucleic acid fractions and proteins were determined using an integrating micro-densitometer (Type GN 2, Barr & Stroud Ltd., Glasgow, U.K.) the commercial version of an instrument designed by Deeley (1955).

In order to calculate the relative fractions of dyestuffs bound to DNA and RNA respectively, preparates were treated with RNase (1 mg/ml 2 hr at 37°C). Further a coverslip was incubated in the same way with distilled water, since the incubation with DNase may change the staining capacity of the batch [39]. As far as the light absorption by the nucleic acids in this method is expressed in relative working units (AE) this step is necessary in order to evaluate the relationship between coryphosphin-stained working units and the units of gallocyanin. Feulgen-stained cells were measured at 570 nm for RNA and DNA and at 480 nm for DNA content. Naphthol Yellow S-stained cells were measured at 430 nm. Unstained cells absorb light equivalent to 2–4 AE. Technical data: Condensor nA 0·3, Objective nA 1·25, moving aperture 0·7 μ, reproducibility of measurements ±3%.

(7) *Determination of cell-number, cell volume and size of cell nuclei*

This was controlled [40] in a Coulter counter — model F. The samples for cell size determinations contained roughly $10^5$ cells per ml physiological saline. Instrument settings were attenuation: 0·707, aperture current: 16. Care was taken that pycnotic cells were eliminated by shaking the cultures and discarding the supernatant before cell counting. The size of the nucleus was determined

according to the method of Sandritter *et al.* [41]. Feulgen-stained cells were photographed. The magnified (17×) picture was projected onto a drawing plate and the circumference of the cell drawn with a pencil. By planimetry the size of the nucleus was assessed. The total error was ±3% with this method.

## RESULTS

(1) *Cell morphology and growth pattern under influence of ARA-C and thio-ARA-C*

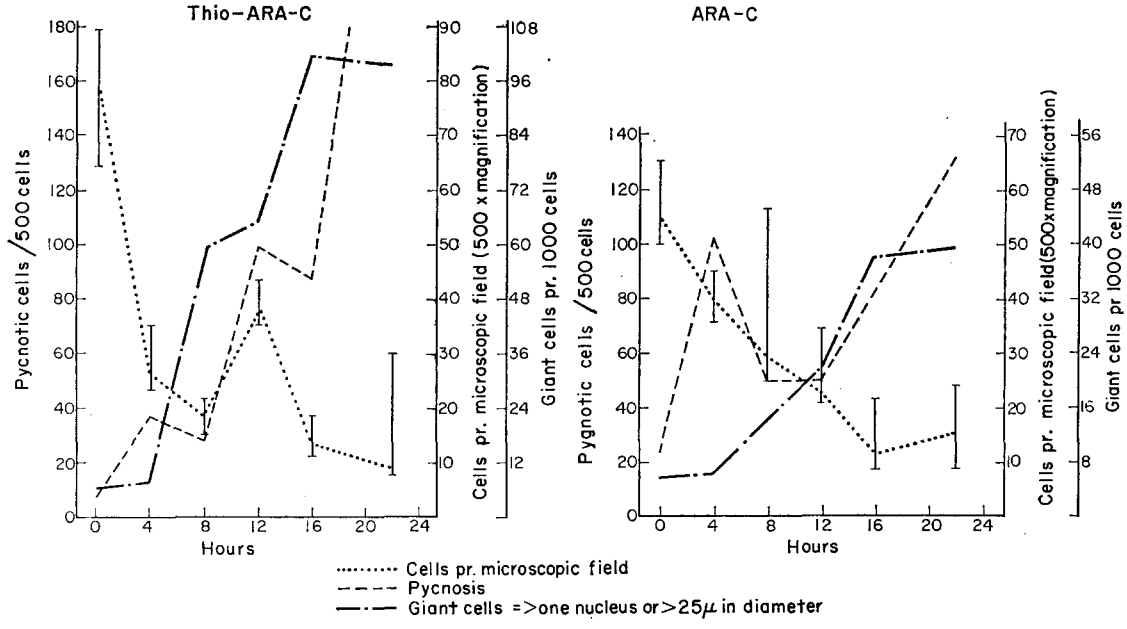
Two diverse effects are encountered: cell death and formation of giant cells.

(A) The appearance of pycnotic cells and the concomitant decrease of cell number can be seen in Fig. 1a and b. The effect on cell viability is more pronounced with thio-ARA-C than with ARA-C. The intensity of cell loss decreased with increasing duration of treatment.

(B) *Mitotic index* — The frequency of mitoses after continuous treatment can be seen from Table 1, while the relative fractions of different mitotic stages are plotted in Fig. 2a and b. Mitosis frequencies less than the control level were observed after 4 hr treatment. At 22 hr no mitoses were seen. Dead cells were of frequent occurrence even after 4 hr treatment, and the appearance of their chromatin material indicated death while in interphase. The chromosomes of the few metaphases seen in treated material were mostly normal and no persistent nucleolar material could be seen. It can be seen from Fig. 2a and b that decrease of mitosis is more pronounced in ARA-C-treated cultures compared with the thio-ARA-C treated.

(C) *Cell enlargement* — The enlargement of viable cells under the influence of ARA-C and thio-ARA-C was encountered shortly after contact with the cytostatics. It was more pronounced in ARA-C-treated cultures until 24 hr when the increase seemingly ceased while the thio-ARA-C-treated cells continued to gain in size up to 40 hr when the observation was stopped. The increase in nuclear size was evident after 12 (ARA-C) and 16 (thio-ARA-C) hr respectively. In both types of cultures vacuoles appeared in the cytoplasm after 24 hr. Both single, large vacuoles and small raylie perinuclear vacuoles were observed.

(D) *Appearance of nuclei* — The most prominent feature of the nuclei in cells treated with either of the two cytostatics was their enlargement. The nuclear membrane was thinned out and finally (40 hr) almost disappeared. The chromatin counting of coarse heterochromatin and fine euchromatin became more

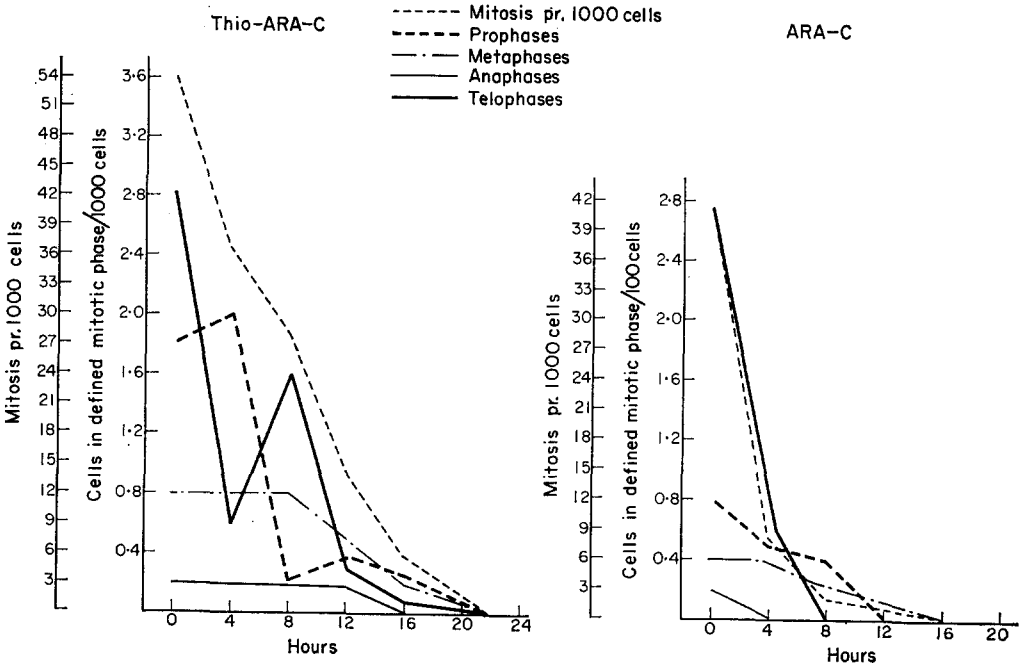



*Figs. 1a and 1b. Effect of ARA-C* 10 μg/ml (1a) *and thio-ARA-C* 10 μg/ml (1b) *on cell number, cell viability and number of giant cells. Cells were grown on coverslips in Roux bottles in the presence of the antibiotics. At selected intervals individual coverslips were fixed in Kahle's fixative and stained according to Feulgen followed by Fast-Green counter staining of the cytoplasm. Number of pyknoses and giant cells were scored in a population of* 1000 *cells/coverslip, while the absolute number of cells/microscopic field was taken as a rough measure of the cell density. Highest and lowest counts are given by the vertical base while the dotted lines connect mean values.*

and more reversed toward finely granular chromatin with increasing time of treatment Finally the chromatin appeared pulverized and only a few remnants of heterochromatin were present.

(E) *Control of cellular volumes* — To substantiate the increase in cellular volume and exclude the possibility that the increase observed in the light microscope could be due to more or less flattening of the cells under the pressure of the coverslips, aliquots of cell suspensions grown in the presence of ARA-C were counted in a Coulter counter model F. As can be seen from Fig. 3, ARA-C effects an approximately 2,5 times increase of cellular volume in 40 hr.

(F) *Planimetric measurements of cellular and nuclear sizes* — The proportion of cytoplasmic to nuclear size in treated cells was estimated by planimetry (Fig. 4). In accordance with the morphologic and the volumetric observations a real increase in cytoplasmic as well as nuclear size was found. Furthermore it looks as if the factor(s) responsible for the cellular enlargement were equally accumulating in the cytoplasm and in the nucleus.




*Figs. 2a and 2b. Effect of ARA-C* 10 μg/ml *(2a) and thio-ARA-C* 10 μg/ml *(2b) on the frequency of total and individual mitotic figures in cultures grown on coverslips in Roux bottles. At selected intervals individual coverslips were fixed and stained according to Feulgen and scored for mitotic figures/*1000 *cells.*

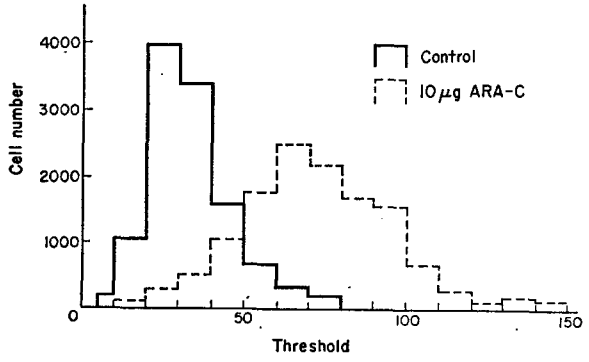



*Fig. 3. Coulter counter-size distributions of controls and cells treated with* 10 μg *ARA-C/ml for* 40 *hr. Cultures were grown in Roux bottles, then trypsinized and resuspended in physiological saline at a density of roughly* $10^5$ *cells/ml. Pyknotic cells were eliminated by shaking the cultures and discarding the supernatant before trypsinization. For further details see Methods.*

(2) *Macromolecular synthesis in L-cells under influence of ARA-C and thio-ARA-C*

When L cells are subjected to treatment with ARA-C or thio-ARA-C, their capacity for synthesizing DNA and RNA is decreased. This inhibition is dependent both on the concentration of the inhibitor and on the duration of the treatment. Figures 5 and 6 demonstrate the

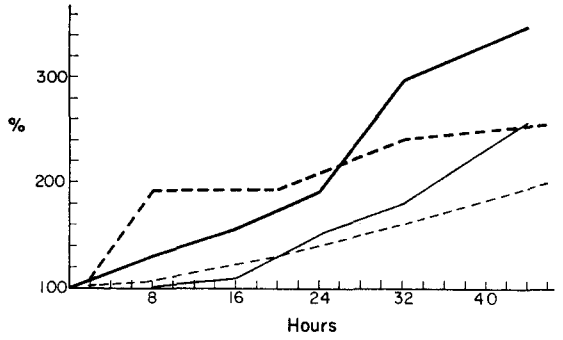



*Fig. 4. Relative total cell-area and relative nuclear area of cells treated with ARA-C or thio-ARA-C 10 µg/ml. Feulgen stained cells were photographed. From the magnified photos (17×) drawings were made of the cellular and nuclear circumference and their areas were measured by planimetry according to* [41]. ——— *relative total cell-area: thio-ARA-C,* – – – – *relative total cell-area: ARA-C,* ——— *relative nuclear area: thio-ARA-C,* – – – – – *relative nuclear area: ARA-C.*

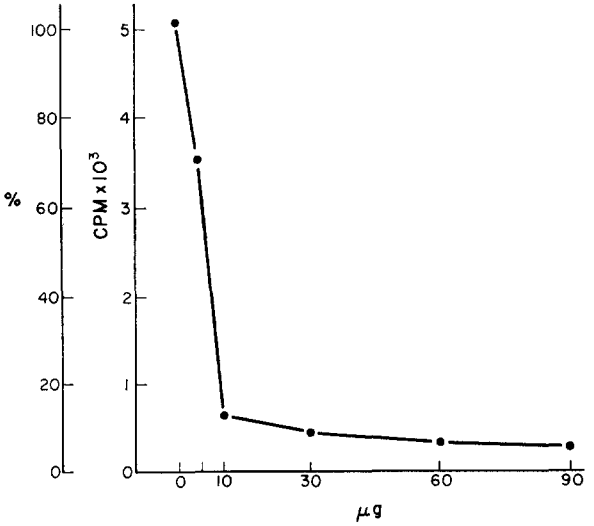



*Fig. 5. Effect of ARA-C 10–90 µg/ml on $^{14}$C-Thd incorporation into acid insoluble material of L cells. For further details see Methods.*

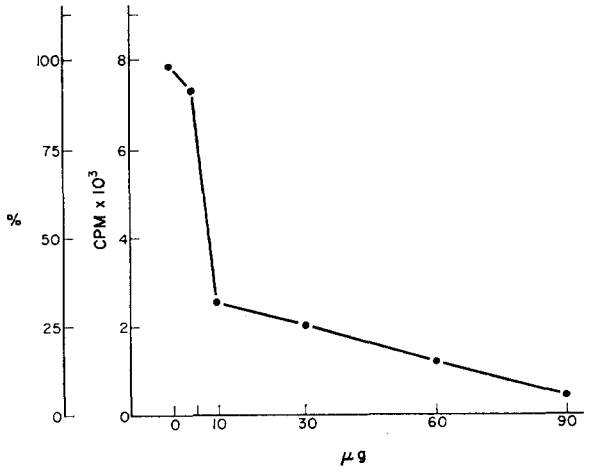



*Fig. 6. Effect of thio-ARA-C 10–90 µg/ml on $^{14}$C-Thd incorporation into acid insoluble material of L cells. For further details see Methods.*

relationships between the concentration of ARA-C and thio-ARA-C and the amount of DNA synthesized when the cells are pulse-labelled after contact with the inhibitors for 2 hr. ARA-C is the more effective of the two compounds, but neither of the two is able to inhibit DNA synthesis completely within the concentration range investigated. Using a concentration of 10 μg/ml one obtains an inhibition of DNA synthesis of 88% (ARA-C) and 70% (thio-ARA-C) respectively. By prolonged treatment of the cells with 10 μg cytostatic/ml the inhibition is found constant for roughly 6 hr after which it gradually declines. After 12 hr RNA synthesis is normal in thio-ARA-C-treated cultures and inhibited to only 25% in cultures influenced by ARA-C. The effects on DNA synthesis are more constant since the compounds have lost only 10–15% of their effect after 12 hr (Fig. 7).

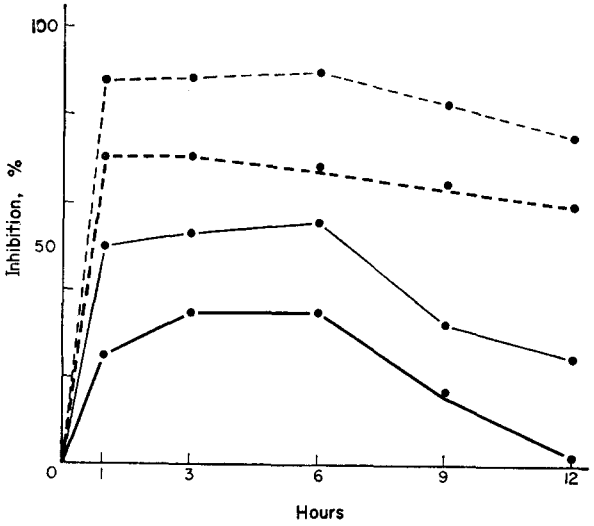



*Fig. 7. Effects of ARA-C or thio-ARA-C 10 μg/ml on the incorporation of $^{14}$C-Thd or $^{3}$H-UdR into acid insoluble material of L cells. For further details see Methods.* ——— *thio-ARA-C/$^{3}$H-UdR,* ——— *ARA-C/$^{3}$H-UdR,* – – – – *thio-ARA-C/$^{14}$C-Thd,* – – – – *ARA-C/$^{14}$C-Thd.*

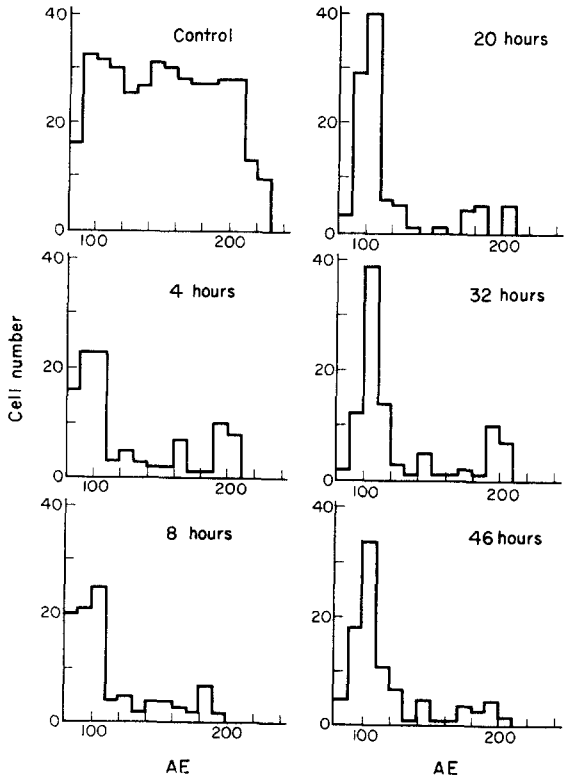



*Fig. 8. Histogrammes from cultures treated with ARA-C 10 μg/ml (0–46 hr), stained according to Feulgen and measured in the integrating microdensitometer for absorption at 570 nm. Abscissa: DNA content in arbitrary units (AE), ordinate: cell number. For technical details see Methods.*

(3) *DNA and RNA content of single cells under influence of ARA-C and thio-ARA-C*

Using the integrating microdensitometer it is possible to measure the total amount of DNA, RNA or protein present in a single cell. In doing so, one has to assume that the dye-stuffs

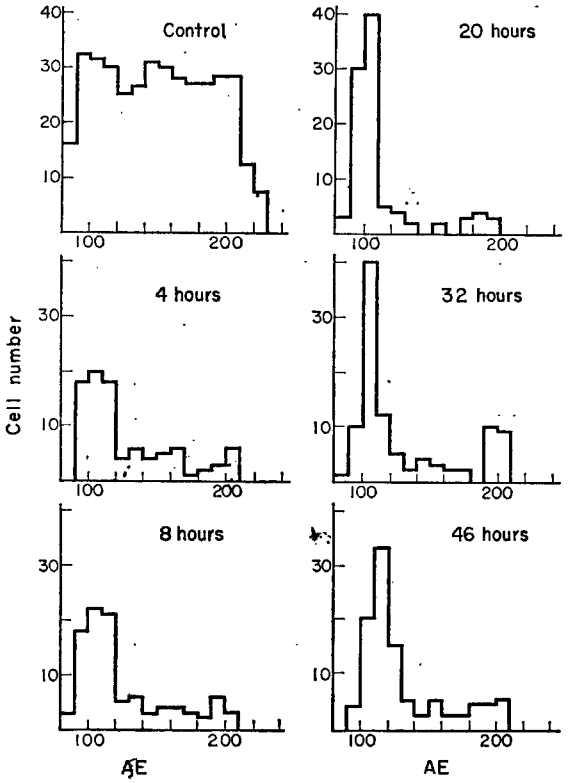



*Fig.* 9. *Histogrammes from cultures treated with thio-ARA-C* 10 µg/*ml* (0–46 *hr*), *stained according to Feulgen and measured in the integrating microdensitometer for absorption at* 570 *nm. Abscissa: DNA content in arbitrary units* (*AE*), *ordinate: cell number. For technical details see Methods.*

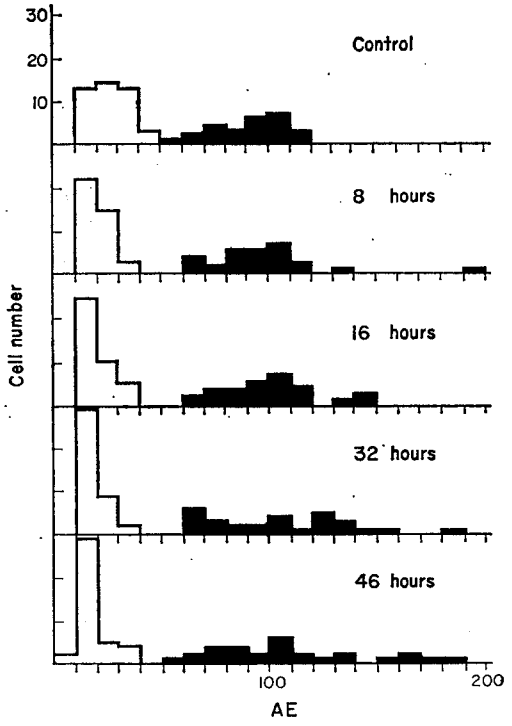



*Fig.* 10. *Histogrammes from coverslip cultures treated with thio-ARA-C* 10 µg/*ml* (0–46 *hr*), *CAGC stained according to Kiefer* et al. [35, 36] *and measured in the integrating microdensitometer for absorption at* 480 *nm and* 570 *nm for RNA and DNA respectively. Open bars: DNA content, black bars: RNA content. For technical details see Methods.*

used act in a specific and stochiometric way with their substrates. For the Feulgen staining, the validity of such assumptions is fairly well-established [13, 42, 43]. Also Deitch's method [38] is well established. The CAGC stain [36] is new, but since it seems to be the only staining method in which both DNA and RNA can be measured in the same cell, we decided to use it and to compare the results obtained in this way with those obtained from Feulgen-stained cells. From the histograms in Figs. 8 and 9 it can be readily seen, that the two cytostatics have identical effects on the composition of the cultures. The main effect seems to be a killing of cells in the S-phase and a partial block between $G_1$ and S. However, a small part of $G_1$ cells seems to be able to proceed through S and reach $G_2$ where they are trapped, creating a $G_2$ peak. These effects are seen in CAGC-stained cells too, although less clearly. In

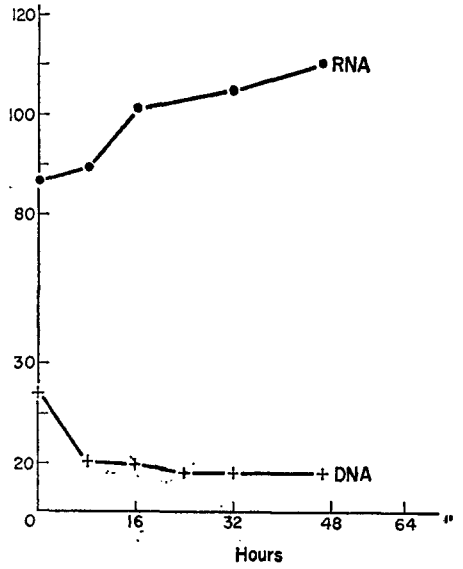



*Fig.* 11. *Plotting of mean values of the histogrammes presented in Fig.* 10. *Upper curve: RNA working units. Lower curve: DNA working units.*

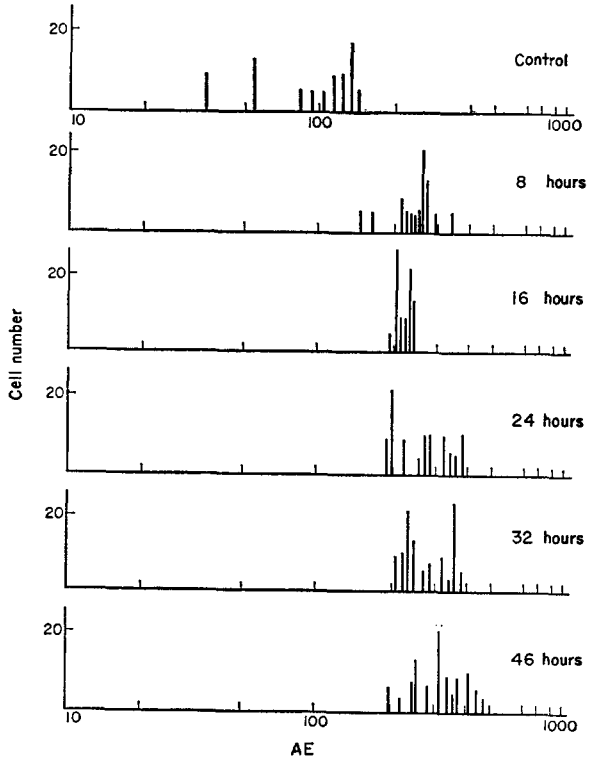



*Fig.* 12. *Histogrammes of cultures treated with ARA-C* 10 μg/ml (0–46 *hr*), *stained for proteins according to Deitch and measured in the integrating microdensitometer for absorption at* 430 *nm. For technical details see Methods. Abscissa: protein content in arbitrary units (AE), ordinate: cell number.*

accordance with the biochemical data, the RNA content of the cells is seen to increase by about 25% (Figs. 10 and 11). In contrast to this slight increase the content of proteins in the cells increases very rapidly and reaches a peak value of 300% of control after 46 hr (Fig. 12).

(4) *Activity of specific enzymes under influence of ARA-C*

In cell cultures treated with ARA-C the population consists almost exclusively of $G_1$-phase cells. In such cells the activity of dCMP-deaminase, thymidine-kinase and DNA-polymerase are low [34, 44, 45] while the activity of CDP-reductase is constant throughout the cell-cycle [46]. In the last third of the $G_1$-phase, all three low-activity enzymes are induced just before the beginning of DNA synthesis [34, 44, 45]. In ARA-C-treated cells one finds not only an induction of dCMP-deaminase, but an enhancement of the enzyme activity which steadily increases to values about 300% that of controls (Fig. 13) after 48 hr treatment. Furthermore, if one tries to add ARA-C to synchronous cells, the effect will be greater the later in the cell-cycle the cytostatic is added

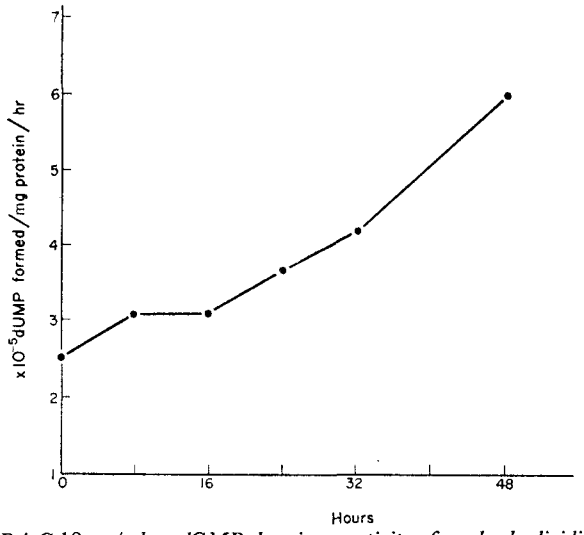



*Fig. 13. Effect of ARA-C 10 µg/ml on dCMP-deaminase activity of randomly dividing L cells treated for 0–48 hr. For further details see Methods.*

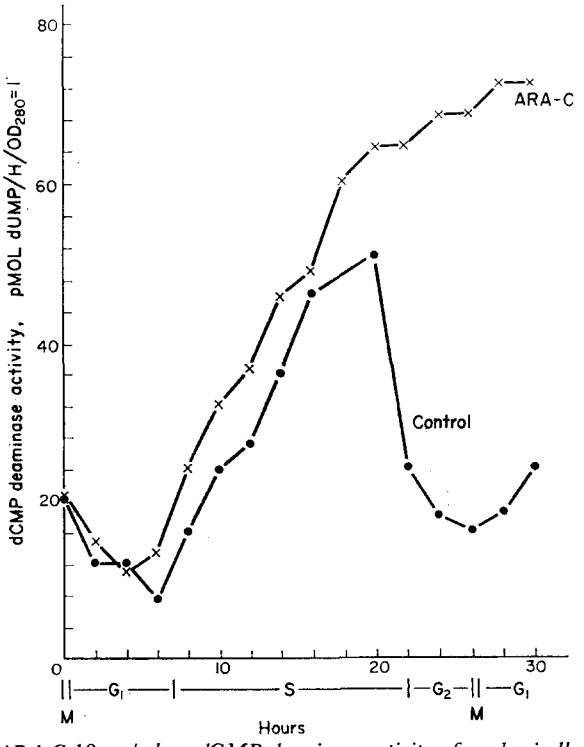



*Fig. 14. Effect of ARA-C 10 µg/ml on dCMP-deaminase activity of mechanically synchronized L cells in subsequent phases of the cell cycle. For further details see Methods.*

(Fig. 14). In L cells DNA-polymerase is found in a soluble and a particle bound form [34]. Under the influence of ARA-C, the gross activity of DNA-polymerase (soluble and particle bound) remains constant, while the proportion of soluble to particle bound-enzyme activity decreases by about 50% after 2 hr treatment [34].

## DISCUSSION

In cells treated with cytosine analogues, at least two cytologically distinguishable processes occur: cell death and formation of giant cells.

At low concentration of cytostatics cell death could be attributed to the inhibition of CDP-reductase [23] and the resulting depletion of cytidine–nucleotide pools, which in turn hamper the DNA-polymerase system. The competition between deoxycytidine and ARA-C for deoxycytidine–kinase, which seems to prefer its natural substrate [10, 18, 20], may result in uncontaminated deoxycytidine-nucleotide pools, however small, and may prevent the DNA-polymerase from building wrong copies.

At higher concentrations of the cytosine analogues, cultures treated for 8 hr or more consist of $G_1$-phase cells (Figs. 8 and 9). In these cells (Fig. 15), the cytostatics presumably (1) block the CDP-reductase but (2) seem to be able to compete with CdR for the CdR-kinase [10, 11] as might be suspected from a substrate competition. Hereby (3) the DNA-precursor pools become contaminated [25]. (4) Through the stimulation of TTP-precursors producing $G_1$-phase enzymes [44, 45] (Figs. 13 and 14) which ARA-C as well as thio-ARA-C [45] and other cytostatics [8] effects, TTP is rapidly built up. Thereby the conversion of dCDP to dCTP is blocked [32]. Since, however, no dCDP is formed from CDP a state of pool depletion of dCMP, dUMP, TMP, and TDP is soon developed, and the cell experiences a decreasing offer of TTP to its DNA synthesis. Furthermore, (6), ARA-C changes the conformation of DNA-polymerase from a soluble (active) to a particulate (inactive) form. These three changes: depletion of TTP and dCTP pools and inactivation of DNA-polymerase (7) block the DNA synthesis. This in turn means (8) that cell division subsides (Figs. 2a and b). Since (9) RNA synthesis not only proceeds (Fig. 7) but is enhanced compared to what is normally found in $G_1$-phase L cells [46] and since (10) the cells proceed to store both RNA and proteins (Figs. 10, 11 and 12) a state of unbalanced growth [47, 48] is established. (11) Both cytoplasm and nucleus are involved in this process (Fig. 4) which (12) leads to formation of giant cells. In our cultures two different types of giant cells could be observed after a few hours treatment: multinucleated and uninucleated. Since endomitoses were of relatively frequent occurrence in coverslips from the first 8 hr of treatment these mitoses may account for the mechanism of giant cell formation in the early phases of ARA-C or thio-ARA-C treatment. In later stages no mitosis in giant cells could be observed, although the number of multinucleated giant cells steadily increased. Since clustering in these stages prevails [17], fusion of cells might be responsible for the formation of multinucleated giant cells.

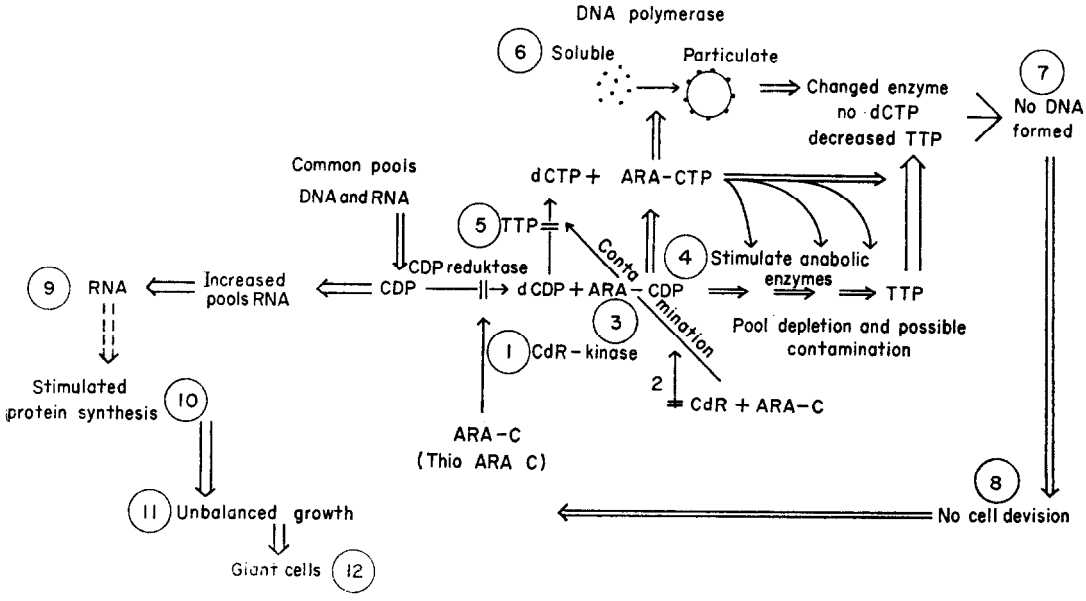



*Fig. 15. Effects of ARA-C (high concentrations) on the synthesis of macromolecules in L cells. For explanatory notes, see Discussion.*

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## SUMMARY

*DNA-synthesis during the life cycle of L cells: Morphological, histochemical and biochemical investigations with ARA-C and thio-ARA-C.*

*Both ARA-C and thio-ARA-C effect a rapid decrease in the mitotic index of L-cells. At the same time giant cells are formed. Volumetric and planimetric measurements show that this is due to equal accumulation of substances in the cyoplasm and in the nucleus. Cytophotometrically the effects of both cytostatics are characterized by selective killing of S and accumulation of $G_1$-phase cells. Also a small accumulation of $G_2$-phase cells is seen, suggesting a block between $G_2$ and mitosis. The giant cells are charaterized by their DNA content as $G_1$-phase cells but contain increased masses of RNA (25%) and proteins (300%). It is shown that one of the enzymes involved indeoxycytidine metabolism (dCMP-deaminase) is greatly enhanced by ARA-C both in randomly and synchronously dividing cells. Simultaneously a shift from soluble to particulate conformation is observed in DNA-polymerase activities. On the basis of these observations combined with earlier investigations on thymidine-kinase and CDP-reductase, a theory for the action of high and low concentrations of ARA-C and thio-ARA-C is proposed.*

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# A New Approach to the Control Mechanisms in Tumor Cells

S. COMOROSAN, S. VIERU and D. SANDRU
*Postgraduate Department of Biochemistry, Fundeni Hospital, Bucharest, Romania*

## INTRODUCTION

THE STUDIES presented in this paper stem from a new type of interaction in biological systems described by us in another context [1].

This interaction was derived on a theoretical basis considering the biochemical substances which may interact in a specific way (substrates or drugs) as "microphysical systems" and the respective specific macromolecules (enzymes or cellular receptors) as quantum "measuring systems". These assumptions are based on Rosen's theoretical contribution concerning a particular quantum approach to the study of biological problems (2–4).

The main experimental result, derived from this type of analysis, is unexpected: when a substrate $S$ of a particular $E_{(S)}$-enzyme is "perturbed" through a very gentle irradiation (up to $10^4$ erg/mm$^2$ of substrate layer) and then tested in the corresponding enzymic reaction; dose-dependent oscillations in the activity of $E_{(S)}$-enzyme are recorded. This proved to be a general effect [5].

A mathematical analysis of these fluctuations indicated the possibility of some interesting "on–off" mechanisms which may be constructed theoretically from out-of-phase oscillations [6]. An experimental argument of this theoretical inference was obtained. Indeed, clear non overlapping oscillations were induced in the activity of a series of enzymes by their respective "perturbed" (i.e. low level irradiated) substrates. Accordingly, a new type of control unit termed the "biochemical flip–flop" was advanced [7].

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The present communication describes the results obtained through this new "perturbation" technique on tumour cells.

## MATERIAL AND METHODS

*Substrates*

The following chemicals (puriss grade level) were used in the substrates "perturbation" (i.e. irradiation) experiments: pyruvate, lactate, glutamate, α-oxoglutarate (Merck) and fructose 1,6 diphosphate (Nutritional Biochemical Co.). The stability of each irradiated substrate sample is checked with current spectroscopic methods (i.r. and n.m.r.-spectra). As expected, for the very low energies utilized in our irradiations, no effects of photolysis could be detected. The irradiated samples were dissolved just before addition to the enzyme assay.

*Irradiation conditions*

The u.v.-irradiations reported here were performed with an high-pressure mercury lamp (no additional interference filters) mounted 30 cm from the designated target plane. The intensity of the radiation at the surface of the powder layer is $3{\cdot}3 \times 10^3$ erg/mm$^2$/min. Samples of 2·5 mg pyruvate, 7·5 mg lactate, 9 mg glutamate, 3 mg α-oxoglutarate and 5 mg fructose 1,6 diphosphate were spread on plastic disks and irradiated in a very thin powder layer. Essentially the same weighed-out unirradiated samples represent the controls.

*Enzymic preparations*

(a) *Purified rabbit liver GDH (glutamic dehydrogenase)-enzyme.* A highly purified preparation is performed according to Strecker [8]. Essen-

tially the same steps in the purification procedure are followed: acetone extraction, acid precipitation, first and second alcohol fractionation, adsorbtion of calcium phosphate gel and crystallization. An enzyme batch of approximately 2000 U/mg protein specific activity is thus obtained.

(b) *Crude FDPase (fructose diphosphatase) and aldolase preparations.* Adult male Wistar rats were killed by decapitation. The livers (or the muscle tissue) were quickly excised and immersed in cold isolation medium: 0·35 M sucrose, 0·05 M tris pH 7·8, 0·07 M KCl and 0·004 M $MgCl_2$. A 20%-initial homogenization is achieved with 0·25 M sucrose. Large tissue fragments are removed by a 2500 *g* centrifugation. The homogenate is then centrifuged in cold for 30 min at 12,000 *g*. The pellet is discarded and the supernatant fluid is further centrifuged in cold at 110,000 *g* for 30 min. The clear supernatant fluid thus obtained is used as a source of FDPase and aldolase.

(c) *Ehrlich ascites cells.* Ehrlich-ascites carcinoma cells were grown for 9–10 days in the peritoneal cavities of adult male Swiss mice of average 25 g weight. An inoculum of $4 \times 10^6$ cells was generally used. The cells were removed with a Pasteur pipette and washed three times by centrifugation and resuspended 1/4 (v/v) in ice-cold isotonic saline. Cells in suspension were tested for viability with 1% trypan blue in normal saline. Only suspensions having less than 0·5% stained cells were utilized in the preparation of crude enzymic extracts.

(d) *Crude enzymic preparations from Ehrlich carcinoma cells.* The cells were resuspended in 9 vol. cold distilled water for 15 min at 4°C. The suspension was then homogenized in glass Potter–Elvehjem homogenizer and centrifuged at 12,000 *g* for 10 min. The clear supernatant is utilized as a source of enzymes.

An extract of hexokinase, purified up to the bentonite step [9], was likewise prepared.

The protein content of the crude enzymic preparations was determined according to Lowry *et al.* [10]. The activity of the dehydrogenases is expressed in units, as recommended by IUPAC [11]. One "enzyme unit" represents the $\Delta A_{340nm}$ in 3 ml of reaction mixture of 0·001 $min^{-1}$ at 25°C, 1 cm light path. Optimal substrate, concentration, buffer ionic strength and pH are stated explicitly for each reaction mixture. Initial velocities ($\Delta_0$) are computed from the progressive curves obtained by readings of absorbancy at 15 sec intervals and are expressed as absorbance changes per min at 340 nm.

The reproducibility of the assays reported here was assessed by performing for each experiment ten replicate determinations with *different enzyme* preparations. The statistical significance of the experimental data was determined by performing, for each point of the figures, ten replicate determinations on *different samples* with the same enzyme batch. Though in the u.v.-irradiations of crystalline layers one would expect a good deal of different absorptions and light scattering, very close values are obtained in replicate experiments with a variance within 2% under the described experimental conditions.

## RESULTS

The main effect of our "perturbation" technique is the following: low-level irradiations (in the range of $10^4$ erg/mm$^2$ of irradiated substrate layer), performed on different biochemical substances, induce a new type of physical $S \rightarrow S^*$ transition. This transition, termed in our context "perturbation", is not detected by current spectroscopic methods (u.v., i.r., e.s.r. and n.m.r.-spectra) but is well "observed" by the corresponding specific macromolecule. This type of experiment revealed a new type of interaction which is the interaction between an $E_{(S)}$-enzyme and its corresponding "perturbed" $S^*$-substrate.

The main result of this interaction is represented by an oscillatory stimulation of the enzymic activity induced with low-level irradiated substrates. This stimulation is dose-dependent. The term "stimulation" stands for an enhancement of the enzymic activity resulting in a more rapid attainment of the steady state. This effect is obtained as well with pure enzymes (Fig. 1) as with purified preparations (Fig. 2) or even crude enzymic extracts (Fig. 3).

The same effect could be demonstrated at the cellular level. A number of enzymic reactions were "stimulated" with a "perturbation energy" derived from typical biochemical energy generating systems like phosphoenolpyruvate-pyruvate kinase, glucose-ATP-hexokinase, creatin-phosphate-creatine kinase instead of the energy derived from the u.v.-irradiations [7].

An example of this type of experiment, performed with a "perturbation energy" derived from the glucose-ATP-hexokinase system of Ehrlich ascites cells is presented in table 1. It is clear that we may now ascribe to this effect a particular biological significance.

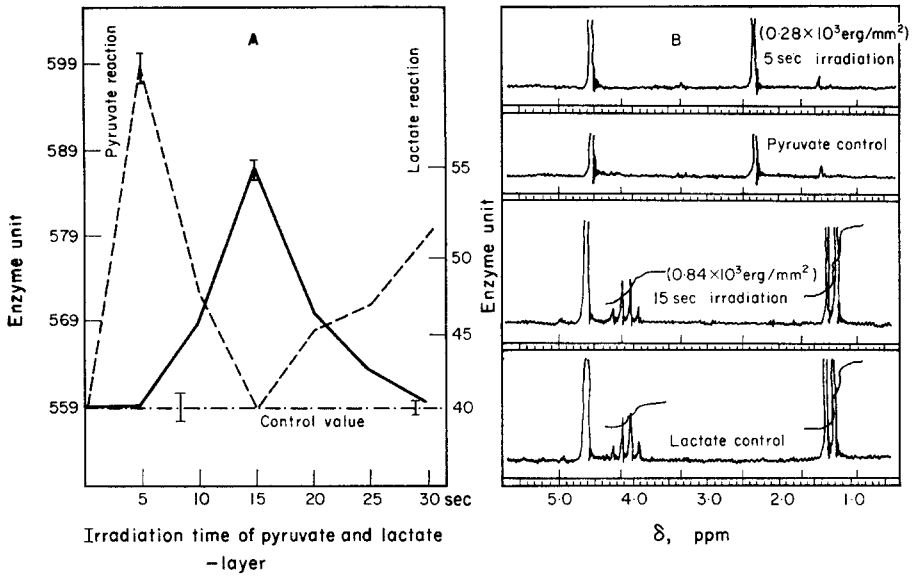


*Fig.* 1. *A. Biochemical "flip–flop" in the pyruvate⇌lactate reaction. Recorded with pure LDH-enzyme. Pyruvate reaction: Na-pyruvate* 2 μ*moles, NADH* 0·52 μ*moles in a final volume of* 2·9 *ml tris buffer* 0·05 M *pH* 7·5. *Lactate reaction: Li-lactate* 16 μ*moles, NAD* 1·5 μ*moles in a final volume of* 2·9 *ml tris buffer* 0·05 M *pH* 7·5. *The reaction is initiated in the spectrophotometer cell with* 6 μg *LDH protein (Schuchardt,* ~20,000 *Bücher Units/mg protein) and followed for* 1 *min at* 25°*C at* $\lambda = 340$ *nm. Pyruvate* (– – –); *lactate* (———). *Each value in the figure is derived from* 10 *replicate experiments performed with the same enzyme batch on different u.v.-irradiated samples. Standard error of control values and of u.v.-irradiated samples inducing a maximum of stimulation is represented. B. High resolution NMR-spectra of the Na-pyruvate and Li-lactate samples, performed on a Varian A-60 spectrometer in* $D_2O$, *at* 25±1°*C, in p.p.m. from TMS as internal reference. The control spectra of the unirradiated substance and the spectra of u.v.-irradiated samples inducing a maximum of stimulation are reproduced.*

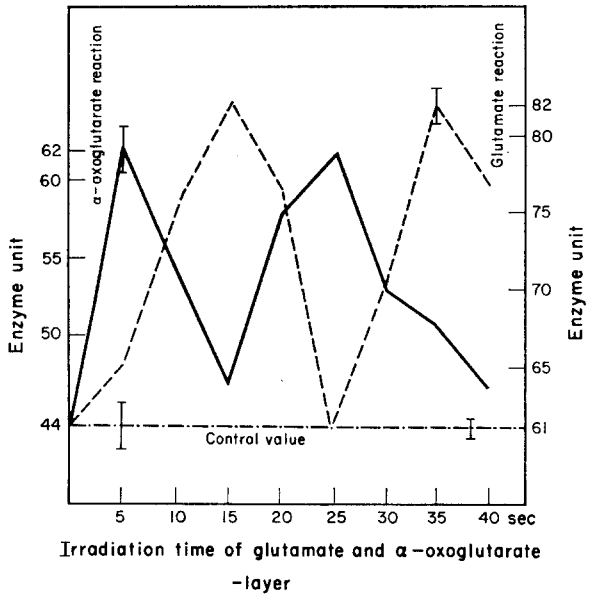


*Fig.* 2. *Biochemical "flip–flop" in the glutamate⇌α-oxoglutarate reaction. Recorded with a purified preparation of rabbit-liver GDH enzyme. Glutamate reaction: Na-glutamate* 10 μ*moles, NAD* 1·5 μ*moles in a final volume of* 2·9 *ml tris buffer* 0·05 M *pH* 7·5. *Glutarate reaction: Na-α-oxoglutarate* 20 μ*moles, ammonium acetate* 20 μ*moles, NADH* 0·52 μ*moles in a final volume of* 2·9 *ml tris buffer* 0·05 M *pH* 7·5. *The reaction is initiated with* 50 μg *protein of rabbit-liver purified GDH preparation, in the spectrophotometer cell at* 25°*C and followed for* 1 *min at* $\lambda = 340$ *nm. Each value in the figure is derived from* 10 *replicate experiments performed with different enzyme proteins. Standard error of control values and of u.v.-irradiated samples inducing a maximum of stimulation is represented. Glutamate* (– – –); *α-oxoglutarate* (———)

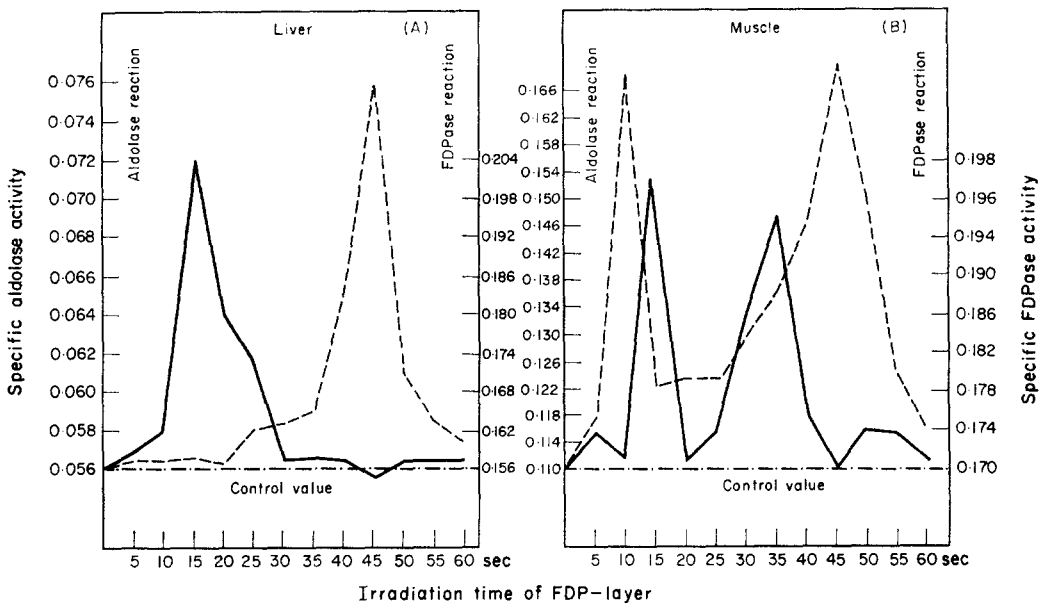


*Fig. 3. Biochemical "flip–flop" in the FDPase⇐fructose 1,6 diphosphate⇒aldolase reaction. A. Recorded with a crude rat-liver preparation. B. Recorded with a crude rat-muscle preparation. FDPase reaction: fructose 1,6 diphosphate-Na 4 μmoles, $MgSO_4$ 5 μmoles in borate buffer 0·05 M pH 9·5. The reaction is initiated with 1 mg crude extract (liver or muscle) protein, followed for 5 min at 37°C, stopped with TCA 10% and the standard colour reaction for the liberated Pi recorded at λ = 720 nm. Specific activity expressed as $\Delta A_{720\ nm} min^{-1}/mg$ protein. Aldolase reaction: fructose 1,6 diphosphate-Na 4 μmoles in tris buffer 0·05 M pH 9·0. The reaction is initiated with 1 mg of crude extract (liver or muscle) protein followed for 5 min at 37°C, stopped with TCA 10%; the colour reaction for the liberated triose developed with 2,4-dinitrophenylhydrazine and recorded. Specific activity expressed as $\Delta A_{540\ nm} min^{-1}/mg$ protein. Each value in the figure is a mean derived from 10 replicate experiments performed with different crude enzymic extracts. FDPase (———); aldolase (– – –).*

Let us now consider an enzymic reaction of the $A \overset{E}{\rightleftharpoons} B$ type. In this case, we may induce an oscillatory variation in the activity of the *E*-enzyme as well with the "perturbed" (i.e. low level irradiated) A-substrate as with the "perturbed" *B*-substrate. In a series of investigated reactions of the above type, clear-cut non-overlapping oscillations were recorded, realizing a "flip–flop" unit [6].

In such a "flip–flop" reaction a certain perturbation level (i.e. a certain irradiation dose) will stimulate the $A \rightarrow B$ flux, leaving the B→A flux unchanged or decreased; conversely, another perturbation level will stimulate the B→A flux, leaving the A→B one unchanged or decreased. A typical example obtained in the pyruvate ⇌lactate reaction, recorded with pure LDH-enzyme and in the glutamate ⇌α-oxoglutarate reaction, recorded with a purified rabbit-liver GDH preparation is presented in Fig. 1 A and Fig. 2.

In an unexpected way this type of biochemical "flip–flop" recorded in the dehydrogenases reactions is *reinforced* by the same out-of-phase oscillations obtained *exactly* at the same irradiation doses, utilizing the respective "perturbed" $NAD^+$ or NADH coenzymes [7].

As may be seen from Fig. 1B no significant changes could be detected in the n.m.r.-spectra of pyruvate and lactate samples, u.v.-irradiated with the energies inducing a maximum of stimulation in the LDH activity.

In a branched reaction, when a substrate *S* is shared by two different $E_1$ and $E_2$ enzymes, the corresponding dose-dependent oscillations induced by the perturbed *S** substrate in the activities of the two enzymes are likewise non-overlapping. A typical example of out-of-phase dose-dependent oscillations induced in the activity of rat-liver (A) and rat-muscle (B) aldolase and FDPase by u.v.-irradiated fructose 1,6 diphosphate is represented in Fig. 3. This series of experimental results led us to investigate these reactions on tumoral cells. It is clear that we may infer on theoretical grounds alterations of these "flip–flop" units if the control mechanisms which regulate the respective metabolic reactions will be impaired. A striking experimental evidence, obtained on Ehrlich-ascites tumor cells confirmed this inference. The pyruvate⇌lactate "flip-flop", recorded with a crude preparation of lactic dehydrogenase from Ehrlich ascites cells, is presented in Fig. 4A. As may be seen from this figure, the control "flip–flop" unit is greatly impaired. Indeed, the dose-dependent oscilla-

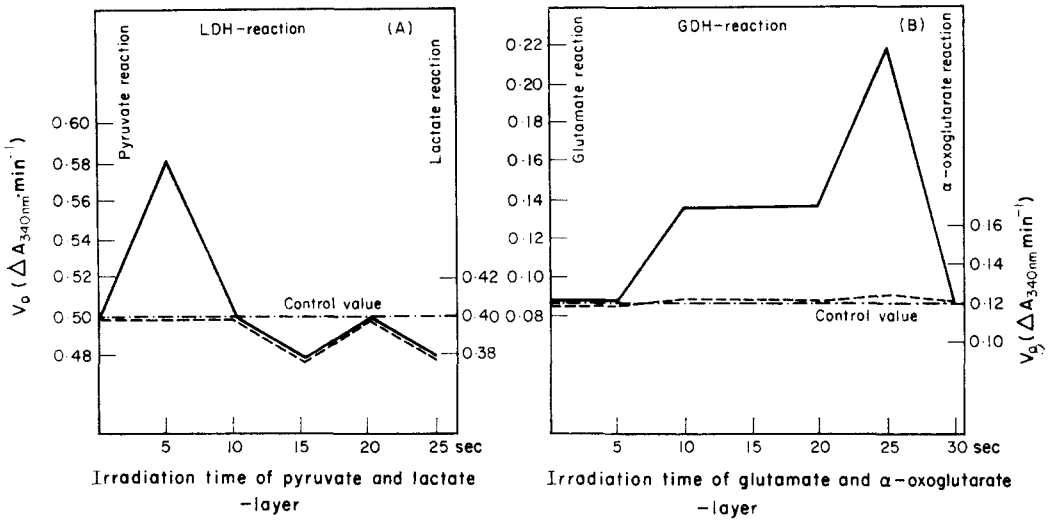


*Fig. 4. Biochemical "flip–flop" in the pyruvate⇌lactate (A) and glutamate⇌α-oxoglutarate (B) reactions, recorded with crude enzymic preparations from Ehrlich ascites cells. Pyruvate⇌lactate-reaction mixture as in Fig. 1. Pyruvate(———); lactate (– – –). Glutamate⇌α-oxoglutarate-reaction mixture as in Fig. 2. Glutamate (———); α-oxoglutarate (– – –). Recorded with 1 mg of crude enzymic extracts.*

tions could be induced in the lactate dehydrogenase activity *only by u.v.-irradiated pyruvate* samples. The enzyme does not "observe" the "perturbation" of the lactate substrate, and similar values of LDH-initial velocity are obtained with the control (unirradiated) samples and with the "perturbed" (irradiated) samples.

The glutamate ⇌ α-oxoglutarate "flip–flop", recorded with a crude preparation of glutamic dehydrogenase from Ehrlich ascites cells is presented in Fig. 4B. The same interesting and unexpected result is obtained. As may be seen from the figure, the dose-dependent oscillations are induced in the glutamic-dehydrogenase activity *only by u.v.-irradiated glutamate* samples. The enzyme is again unable to respond in an oscillatory manner toward *one of its "perturbed" substrates*. The rate of the reductive amination of α-oxoglutarate reaction is not affected by the irradiation of the substrate.

The aldolase–FDPase "flip–flop", recorded with crude enzymic preparations from Ehrlich ascites cells, is presented in Fig. 5. This is a relevant profile. A great peak of aldolase stimulation appears at a very low irradiation energy of the substrate (5 sec equal to $0{\cdot}28\times 10^3$ erg/mm²) corresponding to the situations of intense glycolytic flux. Indeed, the same peak is recorded in the rat-muscle tissue (Fig. 3B) as well at a low irradiation energy of the substrate (10 sec equal to $0{\cdot}56\times 10^3$ erg/mm²), but is delayed in liver tissues (where glucogenesis mechanisms compete with glycolytic mechanisms) to the energy of 45-sec irradiation equal to $2{\cdot}52\times 10^3$ erg/mm² of fructose diphosphate layer (Fig. 3A). Moreover, an additional aldolase-stimulation peak appears at 20 sec irradiation equal to $1{\cdot}12\times 10^3$ erg/mm² of fructose-diphosphate layer.

As can be seen from the figure, the FDPase-stimulation peak is likewise shifted toward lower "perturbation" values (10 sec equal to $0{\cdot}56\times 10^3$ erg/mm² of fructose-diphosphate layer).

## DISCUSSION

The results presented in this paper deserve some special comments. It is clear that low-level "perturbations", realized through low-level irradiations, induce a new type of physical $S\rightarrow S^*$ transition. This transition is characterized by two unexpected physico-chemical properties: (a) it takes place under gentle perturbation energies, contrary to the high energies utilized to obtain "excited states", and (b) it can be stored long enough to produce the observed experimental effects contrary to the short-lived radicals, commonly encountered in high-dose radiation effects. Indeed, using the dehydrogenases substrates, within 70% of the initial stimulation effect is recorded after 24 hr with the u.v.-irradiated powder layer kept overnight at 4°C. In the same context, it is interesting to note the effectiveness and homogeneity in the u.v.-inducement of this $S\rightarrow S^*$ transition, as revealed by the strong statistical significance (very close variance) of the effects observed in replicate irradiated samples.

This brings us to the sophisticated problem of the *physical nature* of the "perturbed" $S^*$ substrate.

A formal mathematical treatment of this aspect, utilizing the concept of *substrate-*

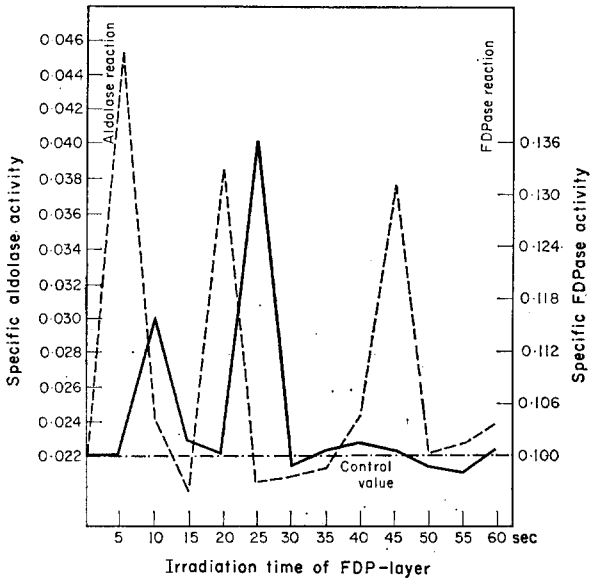


*Fig. 5. Biochemical "flip–flop" in the FDPase←fructose 1,6 diphosphate→aldolase reaction, recorded with crude enzymic preparations from Ehrlich ascites cells. Reaction mixture as in Fig. 3. Recorded with 1 mg of crude enzymic extracts. FDPase (———); aldolase (– – –).*

*enzyme complex entropy* could predict our observed experimental effects [6].

However, it is clear that no classic radiation-induced events of the excitation, free radicals, ionization or molecular rearrangement type may explain the *oscillatory* characteristic of this effect and its surprisingly high *metastability*.

Then, evidently we face a new situation of interaction in physico–chemical systems, in terms of observables which differ from the classic ones. This is a new view-point in modern theoretical biology [12].

Nevertheless, in the present context, the physico-chemical mechanism of our $S \rightarrow S^*$ transition as well as the interaction mechanism between the $S^*$-substrate and the $E_{(S)}$-enzyme remain for the moment an open question.

The oscillatory nature of the effect described by us, raises an important biological problem. Indeed, a number of oscillatory effects obtained under mild irradiations have been reported in a series of phenomena [13]. Likewise, a great number of biological systems exhibiting temporal oscillatory behaviour have been described by Chance [14] and Hess [15] and extended theoretical analyses are available [16].

It seems that in the specific biological reactions, that is in reactions involving mutual recognition, a special oscillatory type of transitions is present. Along these lines, our results clearly suggest that *the enzyme system* is a more sensitive detector of transitions which are induced by very low energies than are the current spectroscopic instruments. Moreover, this type of "detection" is highly specific in different enzymic systems.

The biochemical "flip–flop" unit described by us raises an important control problem. Indeed, an analogy with the allosteric control mechanism may be advanced. Whereas the reaction obtained with the "normal" substrate corresponds evidently to the catalytic effect of the enzyme, the stimulation effect of the "flip–flop" may be analogous to a control effect.

As a typical "stimulation effect" could be obtained with different biochemical energy-generating systems, a biological implication of this phenomenon might be assumed. Moreover, it is clear that our "flip–flop" will induce an oscillation in the substrates concentration, very much like the concentration oscillations of the feedback reactions. The possibility of a cellular control mechanism based on an energy-dependent oscillatory variation of enzymic activities would entail the existence of a cellular source of energy "emitting" well determined impulses in a well determined sequence.

It is not difficult to see that this is exactly the situation involved in the adenylate control hypothesis of Atkinson [17].

The results obtained with crude enzymic preparations from Ehrlich-ascites tumor cells may acquire a particular significance revealing new molecular interactions in important metabolic pathways. Along these lines, the aldolase-FDPase "flip–flop" is an interesting example. The oscillation of aldolase activity at very low substrate perturbation energies and the shift of FDPase-stimulation peak, may provide a molecular model for the intense glycolytic flux

*Table* 1. *"Stimulation" of xanthine-oxidase activity by energy derived from the glucose-ATP-hexokinase (GAH)-energy generating system of Ehrlich ascites cells*

| Reaction medium | $\Delta E_{293\,nm}$/min |
|---|---|
| Xanthine+xanthine oxidase. Control* | 0·128 |
| Xanthine+GAH system (inactivated)+xanthine oxidase. Control | 0·128 |
| Xanthine+GAH system (active)+xanthine oxidase. Experiment† | 0·192 |

*Xanthine-reaction mixture: xanthine 1·5 μmoles in a final volume of 3·0 ml tris buffer 0·05 M pH 7·5. The reaction is initiated with 15 units of xanthine oxidase (Mann Res. Lab.) and recorded spectrophotometrically at 1 min at $\lambda = 293$ nm.

†GAH system: glucose 5 μmoles, $MgCl_2$ 20 μmoles, ATP 1·60 μmoles and partially purified extract of Ehrlich ascites cells hexokinase 50 μg protein in a final volume of 3·0 ml tris buffer 0·05 M pH 7·5. The inactivated GAH control contained the respective hexokinase inactivated for 10 min at 90°C.

The experiment was performed as follows: the GAH system is started with hexokinase at 25°C, run for 5 min, then the xanthine is added, incubated with the system for 3 min and then the enzymic reaction is initiated with the corresponding xanthine oxidase and recorded spectrophotometrically at 1 min. The control experiments were performed essentially in the same conditions with inactivated hexokinase in the GAH-system.

of tumor tissues. Likewise, the loss of lactic dehydrogenase capacity to respond with a "stimulation effect" toward the u.v.-irradiated lactate would shift the equilibrium of the LDH reaction. Indeed, in the pyruvate⇌lactate "flip–flop" reaction (Fig. 1) the stimulation induced in LDH activity by the lactate sample u.v.-irradiated with $0{\cdot}84 \times 10^3$ erg/mm² would represent an accumulation of $2{\cdot}5 \times 10^{-3}$ μmoles pyruvate/min in the transformation of lactate when compared with the transformation of the corresponding unirradiated molecules. This unexpected result may suggest a series of new ideas concerning the investigation of the macromolecular structure of LDH izoenzymes in different tumor tissues as compared with the normal tissues from which the tumor derives.

In this context, the intriguing action of Riley's plasma enzyme-elevating virus [18] may be fruitfully investigated through the lactate and aldolase-FDPase "flip–flops" to account for the LDH elevation and aldolase variable activity. The "stimulation" of the GDH activity in the oxidative deamination of glutamate is an expected result for tissues with intense deamination reactions. The loss of glutamic dehydrogenase capacity to respond oscillatorily in the reductive amination of α-oxoglutarate reaction is another relevant aspect. Indeed, this effect may be well explained as the rate of Krebs cycle substrates oxidation is very slow in tumoral cells. Along these lines, this result may acquire a particular significance in the light of recent biochemical evidence for the possibility of *unidirectional* inhibition of glutamate-dehydrogenase activity in the reductive amination of α-oxoglutarate reaction [19].

Eventually we want to emphasize the fact that the results obtained through our "perturbation" technique in enzymic systems may open a new type of approach to control mechanisms. Many conflicting reports concerning the enzymic activities in tumor cells may be analyzed and reappraised in this context.

## SUMMARY

*A new type of "perturbation" technique for the study of cellular control mechanisms is presented: when a substrate* S *of a particular* $E_{(S)}$*-enzyme is "perturbed" through a very gentle irradiation, and then tested in the corresponding enzymic reaction, dose-dependent oscillations in the activity of* $E_{(S)}$*-enzyme are recorded. In a series of reactions of the* $A \overset{E}{\rightleftharpoons} B$ *type or in a series of branched reactions of the* $P_2 \overset{E_2}{\longleftarrow} S \overset{E_1}{\longrightarrow} P_1$ *type, the oscillations induced by the corresponding "perturbed" substrates in the activity of the respective enzymes were found to be non overlapping. A typical biochemical "flip-flop" is thus obtained. The pyruvate⇌lactate, glutamate⇌α-oxoglutarate and aldolase-FDPase "flip-flop" reactions realized with low level u.v.-irradiated substrates were investigated on Ehrlich-ascites tumor cells. Unexpected results were recorded. In the pyruvate⇌lactate reaction, the oscillations in the LDH activity could be induced only by u.v.-*

*irradiated pyruvate, but not also with u.v.-irradiated lactate. The accumulation of lactic acid by tumoral tissues may be analyzed in this context. Likewise, in the glutamate⇌ α-oxoglutarate reaction, the oscillations in the GDH activity could be induced only by u.v.-irradiated glutamate, but not also with u.v.-irradiated glutarate. In the aldolase-FDPase reaction, the oscillations are induced in the aldolase activity at very low-"perturbation" (i.e. irradiation) energies of fructose* 1,6 *diphosphate, providing a molecular model for the intense glycolytic flux of tumor tissues.*

*The relevance of the new type of approach for the study of control mechanisms in tumor cells is discussed.*

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# Regression of Generalized Leukaemia in Rat Induced by the Granulocytic Chalone

T. RYTÖMAA and KYLLIKKI KIVINIEMI

*II Department of Pathology, University of Helsinki, Haartmaninkatu 3, Helsinki 29, Finland*

## INTRODUCTION

IT HAS BEEN demonstrated recently, both *in vivo* and *in vitro*, that many types of malignant cells can respond by mitotic inhibition to the chalone of their tissue of origin [1–7].

When extracts containing melanocyte chalone [2, 4] were injected for 5 days into mice and hamsters bearing malignant melanomata, all tumours regressed [7], and when extracts containing granulocytic chalone [5, 8, 9] were injected into rats with subcutaneous chloroma, these tumours also regressed and in some cases there was a permanent cure [10]. In all these tumours the events were apparently identical: during the treatment the tumours became soft and necrotic, regressed and ulcerated, and finally the wound healed completely.

In the present study attempts have been made to treat a generalized rat granulocytic leukaemia by means of the granulocytic chalone. These studies have also been supplemented with results obtained *in vitro*.

## MATERIAL AND METHODS

*Granulocytic chalone.* The chalone was obtained from the following sources: (i) rat granulocytes from an aseptic inlammatory exudate [8, 11], (ii) subcutaneous chloroma tumours in rat [5], (iii) whole bovine blood, (iv) blood granulocytes of cow and man, and (v) leukaemic cells of man (chronic myeloid leukaemia).

The preparative techniques used [cf. 5, 8, 11] were essentially similar in all cases. Because most experiments were made with materials obtained from bovine-blood granulocytes, the procedure is described in detail with respect to this source: heparinized bovine blood (50–100 mg heparin per 1 l. of fresh blood) was collected and 2·3 volumes of chilled 0·2% saline was added to haemolyse the red cells, followed after 20 sec by concentrated saline to restore isotonicity. The leucocytes were then centrifuged at 600 *g* for 10 min and the haemolysis procedure was repeated if necessary. The leucocytes, which remained unbroken, were collected (enrichment of granulocytes to about 70% of all leucocytes; [cf. 12]) and were extracted by washing with physiological saline (or Hanks' BSS) at 37°C for 1–2 hr (about 200,000 leucocytes/mm$^3$). The washings were repeated 2–3 times and the clear supernatants were obtained after centrifugation at >3000 *g* for 20 min. The combined supernatants were dialyzed overnight at 4°C (Thomas dialyzer tubing, pore size 4–8 mμ) against distilled water and the dialysate containing granulocytic chalone (molecular weight 2000–4000 [5, 8]) was then lyophilized and stored at −20°C. Before use the lyophilized material was dissolved in water and sterilized by passage through a 'Millipore' filter.

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Besides salt the lyophilized material contains substances which have been released from the unbroken cells in physiological saline and which are small enough to pass through the dialysis membrane. Disc electrophoresis of this material in polyacrylamide gel at pH 8·6 gives 4 or 5 polypeptide bands. If the material is subjected to ultrafiltration on a Pellicon membrane with a cut-off range of 750–1250 the inhibitory activity of the material (according to *in vitro* assay ) is retained by the filter. Disc electrophoresis of the active retentate reveals two distinct polypeptide bands.

*It is thus emphasized that the results reported in the present study have not been obtained by means of a pure and chemically identified granulocytic chalone.* However, because granulocytic chalone is characterized in terms of biological activity [5, 6, 8–11] rather than in terms of chemical properties of the active substance, we feel it justified to use this expression for the crude preparations tested in this study.

In vitro *cultures.* The short-term assay system used *in vitro* has been described in detail elsewhere [5, 8, 11, 13, 14].

In vivo *treatment of chloroleukaemia.* The same subline of transplantable Shay's chloroleukaemia was used as in the previous study on subcutaneous chloroma [10]. The leukaemia, when it was received from Dr. W. C. Moloney, Boston, was in the 134th generation of the original Shay chloroma. The characteristics of this tumour have been described in detail [15–18].

A generalized leukaemia was induced in baby rats of Sprague-Dawley strain at the age of 1–2 weeks by injecting $0{\cdot}9–1{\cdot}6\times10^6$ leukaemic cells into the peritoneal cavity. It may be noted that in several hundred rats up to the age of 15 days transplantations were invariably successful and spontaneous remissions have not been seen.

Before the start of an experiment the leukaemia was allowed to progress for from 1–13 days. In each experiment a homogeneous group of rats (same litter, transplanted simultaneously with the same number of leukaemic cells) was randomly divided into two groups and the treated group was selected by tossing a coin. Control and experimental animals were housed together.

The main attempts to treat chloroleukaemic rats were made with lyophilized material obtained from the granulocytes in 1 l. of bovine blood and dissolved in about 30 ml of distilled water. One to 3 ml of this solution was injected intraperitoneally in a 30 g rat 2–3 times a day, usually every second day. The treatment was continued for periods up to 3–4 weeks.

Control animals were similarly injected with either Hanks' solution or with dialysates prepared from rat liver. Because the survival times of these control rats were not detectably different, the type of the treatment they received has not been indicated separately in the results. It may be noted that all control animals died from leukaemia and that none of them lived longer than 24 days.

*Protective treatment with antibiotics.* Granulocytic chalone causes a serious suppression of normal granulocyte production *in vivo* and this is sometimes followed by a lethal bacterial infection [10]. However, when chalone treatment is discontinued, normal granulocyte production is resumed without the need for bone marrow transplantation. In order to protect the chalone treated animals against bacterial infection, penicillin and streptomycin were injected; the average daily doses given (usually every second day) were 25,000 units of penicillin and 50 mg of streptomycin per kg of body weight. Control animals received similar doses of these antibiotics.

## RESULTS

In vitro *studies*

The purpose of the studies *in vitro* was to determine whether granulocytic chalone is present in the normal granulocytes of rat, cow and man, and in leukaemic cells from rat (chloroleukaemia) and man (chronic myeloid leukaemia), and if so whether the chalone from all these sources inhibits DNA synthesis in the granulocytic progenitor cells of normal-rat bone marrow and in leukaemic cells from both rat (chloroleukaemia) and man (chronic myeloid leukaemia).

*Normal-rat bone marrow cells as target tissue.* The presence of chalone in normal granulocytes of cow and man, and in leukaemic cells of man is shown in Tables 1 and 2.

The results demonstrate that, in each experiment, there was an inhibition of DNA synthesis in the presence of chalone, even if the reactions were not statistically significant at all time points (the overall differences both in Table 1 and in Table 2 are significant at a level $P< 0{\cdot}01$.) It is also apparent from the autoradiographic analysis (Table 1) that chalone caused a decrease in the number of labelled cells and that this effect was mainly, if not solely, due to the response of the granulocytic precursor cells. Thus the results are in full agreement with those obtained previously [5, 8, 9].

*Table* 1. *Response* in vitro *of normal-rat bone marrow cells to granulocytic chalone obtained from leucocytes of bovine blood and from human leukaemic leucocytes (chronic myeloid leukaemia)*

| Incubation time (hr) | Counts per minute | | | | |
|---|---|---|---|---|---|
| | | Chalone from | | | |
| | Control | bovine leucocytes | INH (%) | human leukaemic leucocytes | INH (%) |
| 3 | 1240±85* | 726± 10 | 41 | 856± 17* | 31 |
| 6 | 1958±41* | 1455± 99 | 26 | 1293± 69* | 34 |
| 24 | 1072±60 | 658±129 | 39 | 1314±132 | — |

| | Control | Chalone | INH (%) |
|---|---|---|---|
| All labelled cells | 20·3 | 17·7 | 13 |
| Labelled granulocytic cells | 18·7 | 12·6 | 33 |

*Subject to autoradiographic analysis: labelling indices
The counts (mean±SE) indicate total incorporation of $^3$H-thymidine in the target cells in four replicate cultures at each time point.

*Table* 2. *Response* in vitro *of normal-rat bone marrow cells to granulocytic chalone obtained from blood leucocytes of normal man*

| Incubation time (hr) | Counts per minute | | |
|---|---|---|---|
| | Control | Chalone | INH (%) |
| 2 | 2473± 53 | 1770±156 | 28 |
| 4 | 3226±303 | 2134±187 | 34 |
| 7 | 2890±321 | 2458±106 | 15 |
| 21 | 1698±206 | 2078±241 | — |
| 24 | 1545±424 | 1602±105 | — |
| 3 | 2585± 23 | 2084± 45 | 19 |
| 5 | 2624±131 | 2152±103 | 18 |
| 7 | 2603±101 | 2504±114 | 4 |
| 21 | 1988±101 | 1695± 37 | 15 |

The counts (mean±SE) indicate total incorporation of $^3$H-thymidine in the target cells in four replicate cultures at each time point (two independent experiments).

*Rat leukaemic cells as target tissue.* The response of chloroleukaemic cells of rat to the granulocytic chalone is shown in Tables 3 and 4.

The results demonstrate that chalone obtained from normal granulocytes of cow and from chloroleukaemic cells of rat caused distinct inhibition of DNA synthesis in the target cells, and that the effect was dose dependent. Determination of absolute cellularities in the cultures treated with 'chloroleukaemic chalone' (Table 4) suggests that the higher dose used may have caused a small decrease in the num-

*Table* 3. *Response* in vitro *of chloroleukaemic cells to granulocytic chalone obtained from leucocytes of bovine blood*

| Incubation time (hr) | Chalone dose | | | | |
|---|---|---|---|---|---|
| | Control | 10% | INH (%) | 20% | INH (%) |
| 2 | 332± 52 | 387±36 | — | 262± 7 | 21 |
| 4 | 528± 26 | 490±48 | 7 | 433±25 | 18 |
| 8 | 1049± 61 | 933±60 | 11 | 602±56 | 43 |
| 23 | 901±121 | 682±92 | 24 | 491±32 | 46 |

The counts (mean±SE) indicate total incorporation of $^3$H-thymidine in the target cells in four replicate cultures at each time point. The chalone doses (10 and 20%) indicate relative volumes of the test solution added to the cultures at zero time. Note that the basic components of the test solution and the culture medium were the same (Hanks' BSS).

*Table 4. Response* in vitro *of chloroleukaemic cells to granulocytic chalone obtained from chloroleukaemic cells*

| Incubation time (hr) | Control | Chalone dose 15% | INH (%) | 30% | INH (%) |
|---|---|---|---|---|---|
| 2 | 1181± 42 | 1032± 19 | 13 | 668± 16 | 43 |
| 4 | 2100±207 | 2057± 70 | 2 | 1314±164 | 37 |
| 16 | 3731±467 | 3561± 74 | 5 | 2365±126 | 37 |
| 20 | 3928± 79 | 3604±226 | 8 | 2254±128 | 43 |
| Cellularities | 434± 15 | 446± 41 | | 384± 20 | |

The counts (mean±SE) indicate total incorporation of $^3$H-thymidine in the target cells in four replicate cultures at each time point. The chalone doses (15 and 30%) indicate relative volumes of the test solution added to the cultures at zero time. Note that the basic components of the test solution and the culture medium were the same (Hanks' BBS). Cellularities (mean±SE) indicate average cell counts in a lane across the cover glasses.

ber of cells attached to the cover glasses, although the difference is not statistically significant. Such a decrease, even if real, is too small to explain the 40% inhibition as observed in DNA synthesis.

*Human leukaemic cells as target tissue.* The response of leukaemic cells of man (two patients with chronic myeloid leukaemia) to the granulocytic chalone *in vitro* is shown in Fig. 1. These results are typical examples from a more extensive study now being made in collaboration with Dr. C. Wasastjerna.

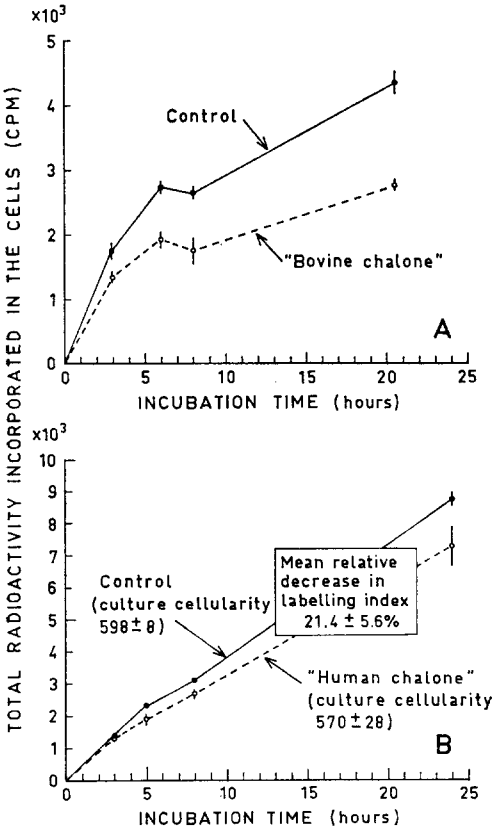


*Fig. 1. Response* in vitro *of leukaemic cells (two patients with chronic myeloid leukaemia) to granulocytic chalone obtained from blood leucocytes of cow (A) and man (B). The counts (mean ± SE) indicate total incorporation of $^3$H-thymidine in the target cells in four replicate cultures at each time point. Culture cellularities (B) indicate average cell counts in a lane across the cover glasses at various time points. The relative decrease (mean ± SE) in the labelling index (B) was obtained from an autoradiographic analysis of the cover glass cultures at each time point (hence $N$=4).*

The results demonstrate that the average inhibition of DNA synthesis was 31·0±2·8% (mean ± SE; $N$=4) in the first case ('bovine chalone') and 11·2±3·6% in the second case ('normal human chalone'). A subsequent autoradiographic analysis from the second experiment (Fig. 1B) indicated that the inhibition was entirely due to a decrease in the number of labelled cells. Determination of labelling indices in the different subpopulations of the leukaemic marrow was omitted, because most of the labelled cells were leukaemic.

*General conclusions.* (i) granulocytic chalone is present in normal granulocytes of rat, cow and man, and in leukaemic cells from rat and man; (ii) granulocytic chalone from any of these sources inhibits DNA synthesis in normal granulocytic precursor cells, and also in leukaemic cells from both rat and man.

## In vivo *studies*

*In vivo* experiments with granulocytic chalone were technically difficult because of the large quantity of material needed. Thus more than 100 l. of bovine blood was required as starting material for the preparation of enough chalone to allow treatment of chloroleukaemia in 40 baby rats (see p. 407). This is the reason why these *in vivo* experiments were done with a relatively small number of animals.

*Effects of granulocytic chalone* in vivo. Eight rats were treated for 4 days with chalone obtained from rat granulocytes and the blood picture was followed for 2 weeks after the cessation of the treatment. Another group of

*Table* 5. *Effect of granulocytic chalone ('rat chalone') on white and red-cell counts of young rats*

| Material injected | Leucocytes/mm³ (mean±SE) | Erythrocytes/mm³ $\times 10^6$ (mean±SE) |
|---|---|---|
| Hanks' BSS | 26 400±2400 | 5·16±0·16 |
| Chalone | 18 500±1300 | 5·83±0·16 |

The values indicate cell counts in eight rats during an observation period of 2 weeks after a 4-day treatment.

*Table* 6. *Effect of granulocytic chalone ('chloroleukaemic chalone') on white and red-cell counts of chloroleukaemic rats*

| Group | Leucocytes/mm³ (mean±SE) | Erythrocytes/mm³ $\times 10^6$ (mean±SE) |
|---|---|---|
| Controls | 24 000±1400 | 2·63±0·20 |
| Treated | 14 300±1000 | 2·62±0·11 |

The values indicate cell counts in three rats after a 9-day treatment (10 days after intraperitoneal transplantation of leukaemic cells).

8 rats, injected with Hanks' BSS, served as controls. The results are summarized in Table 5.

These results demonstrate that red cell production was not inhibited by the chalone injections. In contrast, the leucocyte counts were 30% ($P<0·01$) lower in the chalone treated rats than in the controls (in this experiment, the granulocyte and the lymphocyte counts were not measured separately; cf. however the results given below. The leucocyte values as a whole are somewhat high in both groups, apparently because of a tail infection due to the frequent blood sampling. It should be noted that antibiotics were not given to either group.

Another example of the effect of granulocytic chalone on blood-cell counts is given in Table 6.

These results again indicate that granulocytic chalone had no inhibitory effect on red-cell count but that it caused a 40% ($P<0·01$) decrease in the white-cell count. It may be noted that the severe anaemia of the rats was due to the leukaemia. As in the previous example (Table 5) the white-cell counts were again somewhat high as compared to normal rats of the same age, but in this case the apparent reason was the leukaemia and not a tail infection.

Blood-cell counts alone are inadequate indicators of the inhibitory effect of the granulocytic chalone *in vivo*; confirmation must be sought from studies of the inhibition in the bone marrow. These show that injections of granulocytic chalone for 1–2 days in chloroleukaemic rats caused reductions of 31% in the total DNA synthesis and 24% in the overall labelling index in the bone marrow cells [6].

Another example of the effect of granulocytic chalone on both bone marrow cells and the circulating leucocytes *in vivo* is provided by the results obtained with a few male mice (an inbred CBA strain). Two mice were injected for 5 days with granulocytic chalone ('bovine chalone') while 3 mice, injected with Hanks' BSS, served as controls. Three hours after the last injection each animal received 60 μc of $^3$H-thymidine and 2 hr later the mice were killed. Determination of radioactivities in the bone marrow cells gave the following results (CPM/$10^6$ bone marrow cells): 586, 671 and 795 in the controls, and 230 and 486 in the chalone-treated mice, which indicate a 40–50% inhibition in DNA synthesis. During the 5-day treatment period the blood granulocyte counts in the chalone-treated mice were, on an average, 42% lower than in the controls ($P<0·01$) while the difference in the total blood-leucocyte counts was 14%. Thus the results with inbred CBA mice are consistent with those observed in outbred Sprague-Dawley rats, and they also suggest that the effect of the granulocytic chalone is target tissue specific (note the evident lack of effect on blood-lymphocyte counts).

The above results indicate that the granulocytic chalone acts specifically on the granulocytic system both *in vitro* and *in vivo*. In order to obtain information with respect to tissues other than those in the bone marrow, the effect of a long-term chalone treatment on the growth rate of baby rats was followed in several experiments. A typical example is given in Fig. 2.

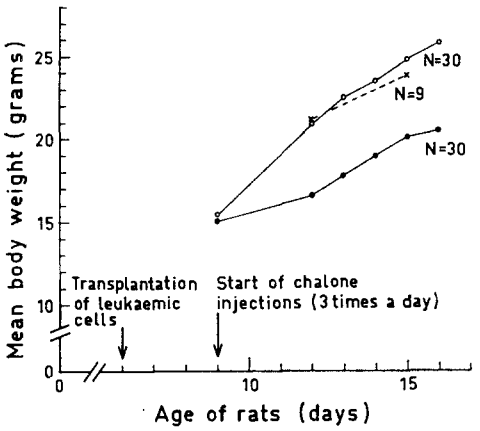


*Fig. 2. Effect of chalone treatment on the growth rate of baby rats.* ●—● *Untreated leukaemic rats;* ○—○ *leukaemic rats treated with granulocytic chalone ('chloroleukaemic chalone');* X---X *untreated normal rats. N is the number of rats weighed at each time point.*

These results demonstrate that the treatment did not retard the body growth. It is therefore clear that granulocytic chalone does not have a generalized inhibitory action in the body; further evidence supporting this conclusion is given below (p. 408).

*Effects of granulocytic chalone on chloroleukaemia.* It has been previously [10] demonstrated that when preparations containing granulocytic chalone are injected into rats with subcutaneous chloroma, these tumours regress and in some cases there is a permanent cure. It is clear, however, that a locally growing tumour differs from a generalized leukaemia in many important respects. Besides the topography of the malignant cells, these local tumours, when small, may sometimes regress spontaneously; in contrast no spontaneous remissions have been seen in the generalized leukaemia. Furthermore, subcutaneous transplantation of chloroma cells is not always successful whereas all intraperitoneal transplantations have been successful in several hundred rats up to the age of 15 days. It may also be emphasized that the generalized leukaemia is biologically more malignant than the subcutaneous chloroma tumours, as indicated by the survival times of the rats. The mean survival time of rats with generalized leukaemia, as estimated from a group of 244 animals, was 12·2 days and the longest individual survival time was 24 days, whereas the survival times of rats with local tumours were about twice as long [10].

In the first 14 experiments with generalized chloroleukaemia, in which either small amounts or impure preparations of granulocytic chalone were used the survival time of the treated rats was prolonged (Table 7), although the mean survival time of all the treated rats ($N$=221) was only 11·2% longer ($P<0·01$) than that of the controls ($N$=202). The longest survival time of the control rats was 24 days while one treated rat lived for 39 days. This rat, treated with material prepared from chloroma tumours, appeared non-leukaemic for 2 weeks after the last control animal in this experiment had died. Subsequently, leukaemia recurred and the rat died not from the disease but as a result of explorative laparotomy.

It may be emphasized that the various groups of rats treated in these 14 experiments are comparable with those treated in the final set of 8 experiments (see below). From these comparisons it becomes clear that injections of tissue extracts *per se* have no curative effect on the generalized chloroleukaemia, because the amount of foreign material injected into the rats was significantly higher in these 14 experiments than in those described below. The amount of foreign material present in the various preparations used was estimated from the u.v.-absorbancy at 280 mμ.

In order to increase the chalone dose without deleterious side effects due to the impurities present, larger quantities of the granulocytic chalone were prepared in a form which was as pure as practically possible. This was achieved by using non-homogenized granulocytes of fresh bovine blood as the source of the chalone

*Table 7. Effect of impure preparations of granulocytic chalone on the survival time of chloroleukaemic rats*

| Source of chalone | Number of rats (controls+treated) | Average effect on survival time (% of controls) |
|---|---|---|
| Rat granulocytes ($N$=3) | 78+ 77 | +11·2 |
| Chloroma tumours ($N$=7) | 85+103 | + 7·8 |
| Whole bovine blood ($N$=4) | 39+ 41 | +19·4 |

Overall mean (weighed)+11·2%.
$P<0·01$.
$N$ is the number of independent experiments. Mean survival time of all control rats was 12·0 days.

(see p. 401). The increase in chalone dose was based on estimates of the number of granulocytes used as the source of the material and also on the *in vitro* effect of this material.

The results obtained in a set of 8 experiments are shown in Table 8 and in Fig. 3.

The results indicate that the leukaemia was inhibited to some extent in all the treated rats (Fig. 3), this conclusion being supported by the prolonged minimum survival time of the treated rats. The average effect on this parameter was 2–3 days which is statistically significant ($P<0{\cdot}01$, according to Wilcoxon's non-parametric test for matched samples).

Of the surviving 18 treated rats shown in Table 8 nine died later from leukaemia. The remaining nine rats (arising from the eight experiments: 0/7, 0/6, 1/6, 1/5, 2/7, 2/5, 1/2 and 2/2; see also Fig. 3) have shown no signs of leukaemia within an observation period of more than a year. It may also be noted that the cured rats, consisting of both males and females, have bred normally.

The curative effect of the treatment was

*Table 8. Effect of the granulocytic chalone on the survival of rats bearing generalized chloroleukaemia*

| Group | Number of survivors | Number of deaths | Total |
|---|---|---|---|
| Controls | 0 | 42 | 42 |
| Treated | 18 | 22 | 40 |

$\chi^2=21{\cdot}66$; $P<0{\cdot}001$.
Data refer to the times after the death of the last control animal in each of eight experiments.

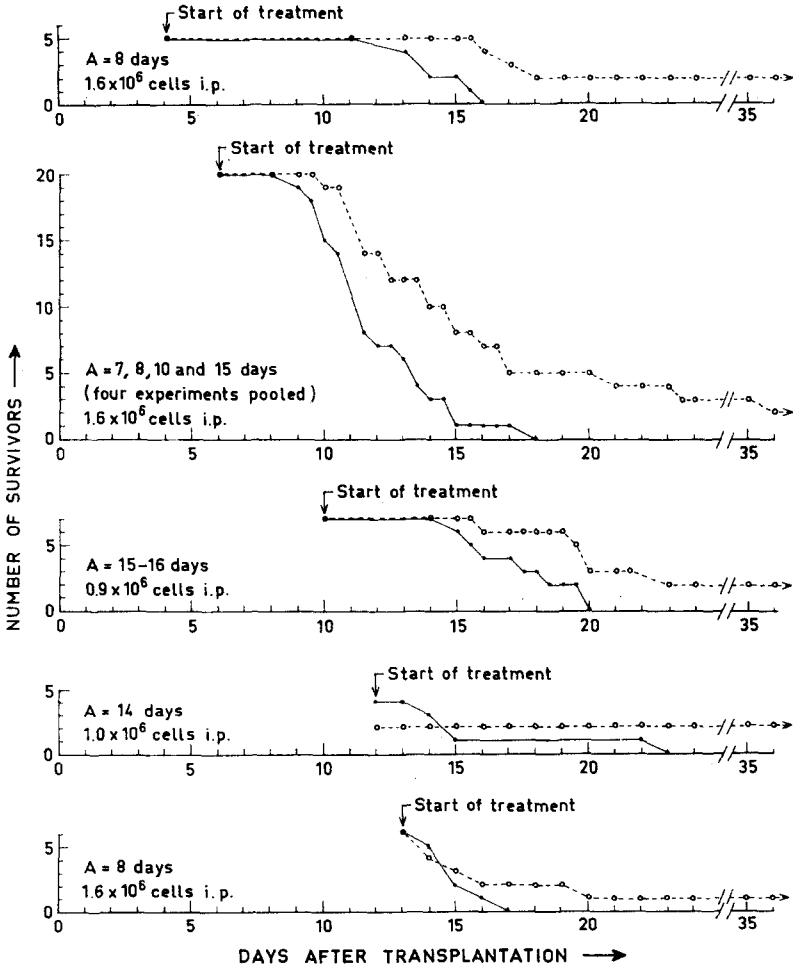


*Fig. 3. Survival times of rats with generalized chloroleukaemia. The five graphs represent results from eight different experiments (experiments are pooled when the start of the treatment was the same).* ●——● *control rats;* ○- - - -○ *treated rats. A=age of rats at the time of transplantation.*

usually obvious to the naked eye after about 10 days: bulging of the abdomen diminished or disappeared (due to the regression of the leukaemic growth in the peritoneal cavity), the ears of the rats began to turn red (due to the relief of the grave anaemia), and fur renewal became active. It was also confirmed that the treatment did not retard the body growth (cf. Fig. 2).

*General conclusions.* (i) granulocytic chalone inhibits DNA synthesis in normal and leukaemic cells, and this inhibition is tissue specific to the granulocytic system; (ii) granulocytic chalone is capable of inducing permanent cure of the generalized chloroleukaemia in rat; (iii) the implication from the *in vitro* results with rat and man and from the *in vivo* results with rat is that cures of leukaemia may also be obtained on man.

## DISCUSSION

The results obtained show that the granulocytic chalone is present in the normal granulocytes of several species of mammal as well as in leukaemic cells from rat (chloroleukaemia) and man (chronic myeloid leukaemia), and that this chalone inhibits DNA synthesis in both normal and leukaemic cells. Thus the granulocytic chalone is not species specific either in respect to its origin or to its inhibitory action. Similar results have been obtained with other chalones. The epidermal chalone has been extracted from a number of species ranging from fish to man, and the preparations obtained have been found to be active on normal epidermal cells of mouse, rabbit and man [19–23] as well as on cells of epidermal carcinoma [1, 24]. Less extensive but essentially similar results have been obtained with the melanocyte chalone [2, 4, 7].

In contrast to the lack of species specificity the inhibitory action of the granulocytic chalone is strictly target tissue specific, as has also been shown before [5, 8]. Essentially similar results have again been obtained with respect to other chalones, such as the epidermal chalone [19, 20, 22], the melanocyte chalone [2] and the sebaceous-gland chalone [25].

However, the most important result of the present study is the demonstration that generalized leukaemia in rat can be completely and permanently cured by means of the granulocytic chalone. It is obviously important to understand the reasons for this reaction.

From the kinetic point of view tumour growth is only possible when the rate of cell production exceeds the rate of cell loss. The obvious question then arises: where is the weakness in the malignant cells?

It has been shown previously [5, 11] that chloroleukaemic cells contain only one tenth or less of the chalone concentration found in normal cells but that despite this the total body content of chalone is significantly higher in the animals bearing chloroleukaemia than in controls. The conclusion is that there must be an abnormally high rate of chalone loss from the leukaemic cells.

It has also been demonstrated here as well as previously [5, 6, 10] that chloroleukaemic cells are capable of responding by inhibition when the chalone content is artificially raised, although the leukaemic cells are less sensitive to the inhibitory action of the granulocytic chalone than are the normal granulocytic precursor cells.

On the basis of these and other similar results [1–4] it seemed realistic to try to treat malignant tumours by means of the tissue specific chalones. The first attempts were made with local subcutaneous tumours and the results exceeded expectations: thus when mice and hamsters bearing large melanomata were treated with the melanocyte chalone all the tumours disappeared [7]. When rats bearing subcutaneous chloroma tumours were treated with the granulocytic chalone their tumours also regressed and often disappeared completely [10]. In many cases the cures were permanent.

The first attempts to treat generalized chloroleukaemia in rats by means of the granulocytic chalone were less successful, but when it became possible to increase the chalone dose (see p. 407), without deleterious side effects due to the impurities present, the results were at least as good as those obtained with local chloroma tumours. Thus the survival time was prolonged in all the treated rats and in 9 animals out of the 40 the leukaemia disappeared completely and permanently.

From a theoretical point of view it may appear strange that tissue specific inhibitors, or chalones, are capable of causing such complete and permanent regression of malignant tumours, and this is especially so because the action of the chalones is not based on a direct destruction of the cells [5, 9, 19]. In fact, in a normal tissue the anti-mitotic action of the chalone is accompanied by a prolongation of the post-mitotic cell life [26].

There are several possible explanations for this seemingly paradoxical situation. One of the most plausible is that malignant cells are not fully responsive to the chalone in that after

mitotic inhibition the post-mitotic cell life is not prolonged; several examples of this kind are given by Bullough and Deol [24]. In such circumstances it is clearly possible to achieve a situation in which the malignant cells have a higher probability of death than of division and this must ultimately result in the regression of the tumour. It may also be noted that the killing of the post-mitotic tumour cells may be partly, or even entirely, due to environmental factors, such as immunological mechanisms [cf. 27] or breakdown of the blood supply [cf. 24).

The fact that normal tissue is not destroyed by the chalone treatment could be a direct consequence of the prolonged cell life caused by the excess of chalone. However, in the granulocytic system the situation differs from that in such tissues as epidermis. Cell recruitment is only partly due to mitosis; it is also due to the entry of cells from the pluripotential stem cell population into the granulocytic system. Thus after the chalone treatment normal granulocyte production recommences rapidly because the stem cells are evidently not inhibited by the granulocytic chalone.

The evidence obtained suggests that higher percentage cures of rat chloroleukaemia can be expected when it becomes possible to give higher doses of the granulocytic chalone. Furthermore, the implication from the *in vitro* results with rat and man and from the *in vivo* results with rat is that cures of leukaemia may also be obtained on man. The problem of larger supplies of purer preparations of the granulocytic chalone is essentially a technical one.

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## SUMMARY

*It has been shown in this study that granulocytic chalone inhibits DNA synthesis in normal and leukaemic cells (chloroleukaemia in rat and chronic myeloid leukaemia in man), and that this inhibition is tissue specific to the granulocytic system. In contrast to this granulocytic chalone is not species specific either in respect to its origin or to its inhibitory action.*

*The most important result of the present study is the demonstration that generalized leukaemia in rat (chloroleukaemia) can be completely and permanently cured by means of the granulocytic chalone. With the maximum dose used in this study the survival time was prolonged in all the treated rats and in* 9 *animals out of the* 40 *the leukaemia disappeared completely and permanently.*

*The implication from the* in vitro *results with rat and man and from the* in vivo *results with rat is that cures of leukaemia may also be obtained on man.*

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# Erythrocyte Osmotic Fragility in Friend Virus-Infected Mice

IRIS B. PARR
*Chester Beatty Research Institute, Clifton Avenue, Sutton, Surrey, England*

K. E. K. ROWSON
*Institute of Laryngology and Otology, University of London, London, W.C.1., England*

MOST STRAINS of Friend leukaemia virus [1] (FLV) cause splenomegaly, anaemia and thrombocytopaenia [2]. The strain we have used causes a very severe anaemia of rapid onset which kills most of the mice in 3–4 weeks, female mice being particularly susceptible [3]. There are strains of FLV which cause a less severe anaemia and one strain which causes polycythaemia [4]. The polycythaemia does not occur in splenectomized mice [4]. The Rauscher leukaemia virus (RLV) [5] causes a disease very similar to FLV except that the animals which do not die in the early acute anaemic phase go on to develop lymphatic leukaemia. RLV causes a reticulocytosis which is dependent on the presence of an intact spleen and the severity of the anaemia in the early stages of the infection is potentiated in splenectomized mice [6].

The severe anaemia which kills mice in the third week of FLV disease is mainly due to haemorrhage into the spleen. Within approximately a week of death, the tissue of the greatly enlarged spleen begins to break down and gradually the spleen becomes a series of blood-filled spaces surrounded by a thin membrane. The slightest trauma to a mouse at this stage can cause splenic rupture. If the spleen does not rupture, the animals die from circulatory failure and anaemia, due mainly to the sudden increase in blood volume with consequent haemodilution. Haemoglobin levels as low as 2·5 g per 100 ml of blood have been observed just before death. This severe anaemia due to blood loss into the spleen only occurs when large doses of RLV are injected. It is prevented by splenectomy, but splenectomy also prevents the reticulocyte response in RLV infection and so the anaemia following moderate doses of RLV is more severe in splenectomized mice [7].

In addition to the anaemia due to blood loss into the spleen, FLV and RLV infected mice have a haemolytic anaemia associated with a reduced erythrocyte survival time [6, 8]. A similar haemolytic anaemia has also been reported in mice infected with the murine erythroblastosis virus [9]. Brodsky and his co-workers [8] concluded from their ferrokinetic and labelled erythrocyte survival experiments that FLV had a direct effect on the erythrocytes in the circulation. In the present studies, osmotic fragility determinations were made in an attempt to detect any abnormality in erythrocytes from FLV infected mice.

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## MATERIAL AND METHODS

*Animals*

Female BALB/c mice bred in the department by brother–sister mating were used when 6 to 8 weeks old.

*Virus*

The strain of FLV used was originally obtained from the Chester Beatty Research Institute, London, S.W.3, and freed from

contamination with the plasma lactate dehydrogenase-elevating virus as previously described [10]. The virus preparations used in these experiments consisted of pooled mouse plasma obtained from FLV-infected mice and diluted 1 in 20 with Hanks' saline containing 0·5% gelatin. Mice were injected with FLV via the retro-orbital sinus [11].

*Test of osmotic fragility*

Blood was obtained from mice under ether anaesthesia by cutting the axillary blood vessels and gently aspirating the blood with a wide-bore graduated pipette. The blood was mixed with Alsever's solution [12], the cells centrifuged down, and washed twice with phosphate buffered saline.

Erythrocyte osmotic fragility was determined by exposing the cells to various concentrations of buffered saline (modified Locke's solution) [13] at pH 7·4 for a standard interval [14]. The unlysed cells were centrifuged down and the free haemoglobin measured in a spectrophotometer at a wave-length of 540 Å.

*$^{51}$Chromium labelling of erythrocytes*

$^{51}$Cr (Radiochemicals, Amersham, Bucks, England), in the form of a sodium chromate solution which contained 9·9 μg Cr per ml, was used to label erythrocytes from normal and FLV infected mice. Erythrocytes collected in Alsever's solution were washed three times with physiological saline and a 50% suspension prepared; 50 μc of $^{51}$Cr were added to 3 ml of a cell suspension which was then incubated at room temperature (20°C) for 1 hr. The erythrocytes were washed three times with saline and resuspended to give a 10% suspension. No radioactivity was detected in the supernatant after the final wash.

## RESULTS

*Fragility under different conditions*

In the first series of experiments, erythrocytes from normal and FLV infected mice were incubated with various concentrations of saline for 30 min at 20°C before the degree of haemolysis was measured and osmotic fragility curves plotted. Under these conditions infection with FLV could not be shown to have any effect on erythrocyte fragility until late in the course of the disease and even then, as shown in Fig. 1, there was virtually no change in the mean saline concentration causing 50% lysis. However, there was a straightening out of the osmotic fragility curve due to an increase in the proportion of erythrocytes both more and less fragile than the average. This change in the shape of the fragility curve suggested that the erythrocytes from FLV infected mice were abnormal and that if they were subjected to a longer period of incubation it might be possible to demonstrate an increase in the mean osmotic fragility. Figure 1 shows the curves obtained when the incubation period was increased to 72 hr at 4°C. Both curves were displaced to the right but the curve for erythrocytes from FLV infected mice was displaced further than the curve for erythrocytes from normal animals.

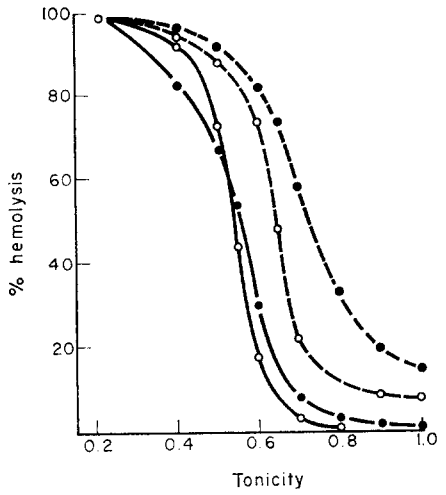


*Fig. 1. Curves showing degree of lysis after incubation of erythrocytes ——— for 30 min at 20°C and — — for 72 hr at 4°C. Erythrocytes from ○ normal mice and ● from mice 19 days after FLV injection. Each curve is the mean of 6 curves for erythrocytes from individual mice.*

Thus erythrocytes from FLV infected mice appear to lyse more readily than normal erythrocytes on exposure to unphysiological stress. Using this modified test it was possible to demonstrate an increase in the mean osmotic fragility of erythrocytes as early as 15 days after the injection of virus, which is about the time anaemia begins to develop. Table 1 shows the mean tonicity causing 50% lysis of erythrocytes from mice 15 and 23 days after FLV injection.

*Table 1. Osmotic fragility of erythrocytes from normal and FLV infected mice*

| Days after virus injection | Tonicity causing 50% lysis* |
|---|---|
| 0 | 0·65†±0·01 |
| 15 | 0·68 ±0·02 |
| 23 | 0·74 ±0·02 |

*Degree of lysis estimated after exposure of erythrocytes at 4°C for 72 hr.

†Values are means ± standard deviation of 6 estimations on erythrocytes from individual animals.

*Effect of splenectomy*

Splenectomized mice survived for longer periods after FLV injection than did intact mice. Splenic haemorrhage was prevented in these mice and the animals finally died 7 or 8 weeks after FLV injection with a severe anaemia and in many cases an enlarged liver.

Splenectomized mice were injected with a small dose ($10^{2.5}ID_{50}$) of FLV as used in the above experiments and erythrocytes osmotic fragility measured by the long incubation method. There was no evidence of increased fragility 23 or 28 days after virus injection. However, after 6 weeks, there was an increased osmotic fragility. Using a larger dose ($10^{5}ID_{50}$) of FLV, which killed intact mice in approximately 15 days, the osmotic fragility curves shown in Fig. 2 were obtained. Splenectomy itself appears to cause a decrease in erythrocyte fragility in uninfected mice and to delay the development of increased osmotic fragility in FLV infected mice. The erythrocytes from intact mice 15 days after the injection of FLV showed a marked increase in osmotic fragility while the erythrocytes from splenectomized mice showed no increase in fragility at this time. However, 23 days after virus injection when the intact mice were dead the splenectomized mice had a very marked increase in erythrocyte fragility.

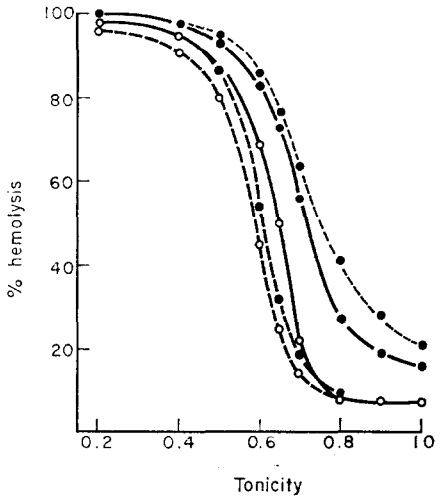


*Fig. 2. Curves showing degree of lysis after incubation for 72 hr at 4°C for erythrocytes from intact and splenectomized mice, FLV infected and uninfected. Each curve is the mean of 6 curves for erythrocytes from individual mice.*
○——○ *intact uninfected;* ○— —○ *splenectomized uninfected;* ●——● *intact FLV infected 15 days;* ●— —● *splenectomized FLV infected 15 days, and* ●- - - - - - ● *splenectomized FLV infected 23 days.*

*Uptake of erythrocytes by the spleen and liver*

Further evidence that the erythrocytes from FLV infected mice are abnormal was obtained using $^{51}Cr$ labelled erythrocytes. Two groups of 6 normal mice were injected intravenously with 0·1 ml of a 10% suspension of labelled erythrocytes. One group received erythrocytes from normal mice and the other erythrocytes from mice infected with FLV for 9 days. After 24 hr both groups of mice were killed, their spleens and livers removed, and the radioactivity estimated on a Packard tri-carb liquid scintillation spectrometer. Table 2 shows the proportion of injected erythrocyte label present in the liver and spleen. The mice receiving erythrocytes from FLV infected mice have, after 24 hr, accumulated nearly twice as much label in their spleens as the mice receiving normal erythrocytes. This increased uptake of erythrocytes from FLV infected mice by normal splenic tissue is compatible with the theory that they are more easily destroyed than normal erythrocytes.

## DISCUSSION

By increasing the incubation period to 72 hr it was possible to show a difference in osmotic fragility between the erythrocytes from normal and FLV infected mice. Whether or not this increased tendency to lyse under unphysiological conditions would render the erythrocytes more liable to destruction in the circulation is uncertain, but in the experiments where labelled erythrocytes from normal and FLV infected mice were injected into normal mice, the label from the FLV infected erythrocytes accumulated in the spleen much more rapidly than the label from normal erythrocytes. This is at least compatible with the view that erythrocytes in FLV infected mice are more

*Table 2. Proportion of injected label in spleen and liver 24 hr after the injection of labelled erythrocytes into normal mice*

| % Injected label in | Source of injected erythrocytes: Normal mice | FLV infected mice | *P* between means |
|---|---|---|---|
| Spleen | 3·5*±0·2 | 6·3±1·1 | 0·001 |
| Liver | 5·7*±0·9 | 7·4=1·0 | 0·01 |

*Values are means ± standard deviation of values obtained in groups of 6 mice.

liable to destruction under physiological conditions than normal erythrocytes.

The displacement of the whole fragility curve to the right in FLV infected mice indicated that the entire erythrocyte population was rendered more fragile. The mechanism for this increased fragility is not clear. Brodsky and his co-workers [8] showed that erythrocytes cohort labelled with $^{59}Fe$ in normal mice had a reduced survival time when the mice were subsequently injected with FLV. Thus indicating that the virus reduces the survival time of normally formed cells. They concluded that the virus had a direct action on the circulating erythrocytes. However, it is unlikely that FLV can replicate in erythrocytes which contain no RNA and there is no evidence that it attaches to the erythrocyte membrane. As an alternative to a direct action of the virus on the erythrocytes, the possibility of a damaging immune reaction similar to that postulated as the cause of splenic necrosis in FLV infected mice [3] was considered, but the direct Coombs' test was always negative in groups of 7 mice tested at different stages of the disease.

In the preleukaemic phase of Rauscher leukaemia virus (RLV) [5] infection, there is an anaemia and thrombocytopaenia [15] with increased erythrocyte fragility [6] similar to that present in FLV infection. An important difference in the effects of RLV and FLV appears when the mice are splenectomized. In the case of RLV the increased erythrocyte fragility is more marked in splenectomized mice whereas in our experiments, splenectomy delayed the onset of increased erythrocyte fragility. The explanation for this difference lies in the reticulocytosis which is produced in the intact mouse by RLV but not by FLV. Reticulocytosis tends to cause a decrease in erythrocyte fragility. After splenectomy reticulocytosis does not occur in the RLV infected mouse.

The spleen does not appear to be an important factor in the decreased survival of erythrocytes in FLV infected mice as Brodsky and his co-workers found that labelled cells were eliminated at a similar rate in splenectomized and intact mice [8]. However, since splenectomy in our experiments delayed the onset of increased erythrocyte fragility, it is possible the spleen may play some part in rendering the circulating erythrocytes more fragile. Intact granulocytes have been shown to cause increased haemolysis of erythrocytes *in vitro* [16] and it may be that haemostasis in the enlarged spleen gives increased opportunity for contact between myeloid cells and erythrocytes, resulting in damage to the latter. In splenectomized mice, the increase in osmotic fragility following FLV injection may result from a similar process taking place in the liver which is enlarged late in FLV disease.

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## SUMMARY

*Erythrocytes from Friend leukaemia virus (FLV) infected mice lysed more readily than erythrocytes from normal mice, on exposure for 72 hr at 4°C to various concentrations of buffered saline. Increased osmotic fragility could first be demonstrated 15 days after FLV injection. In splenectomized mice the onset of increased osmotic fragility and anaemia were delayed but not prevented. When $^{51}$chromium labelled erythrocytes from normal and FLV infected mice were injected into normal mice the erythrocytes from FLV infected mice were taken up by the spleen much more rapidly than the erythrocytes from normal mice.*

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# Structure and Activity in Chemical Carcinogenesis:

## Reactivity and Carcinogenicity of 7-bromomethylbenz[*a*]anthracene and 7-bromomethyl-12-methylbenz[*a*]anthracene

A. DIPPLE and T. A. SLADE

*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W.3*

### INTRODUCTION

A NUMBER of known and postulated metabolites of the aromatic hydrocarbon carcinogens have been tested for carcinogenic activity. After subcutaneous injection, some derivatives of 3-methylcholanthrene, notably 1- and 2-hydroxy-3-methylcholanthrene and 3-methylcholanthrene-2-one, exhibited marked activity in mice [1], whilst all the tested derivatives of 7-methylbenz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene and dibenz[*a*, *h*]anthracene were notably less active than the parent compounds in both mice and rats [2–4]. Two groups of workers have reported that after oral administration, 7-hydroxymethyl-12-methylbenz[*a*]anthracene is of comparable activity to 7,12-dimethylbenz[*a*]anthracene in the induction of mammary tumours in rats [2, 5], whilst a third group [6] found this metabolite to be considerably less active, than its parent in this test system.

It can be seen then, that in contrast to the situation described by the Millers [7] for carcinogenic aromatic amines and amides, no general class of aromatic hydrocarbon metabolite exhibiting greater carcinogenic potency than the parent compound has yet been detected. This has been interpreted by Arcos and Argus [8] as a 'definitive indication that these compounds are active as such without previous metabolic activation'. This argument is not necessarily conclusive, however, since a number of possible mechanisms for the metabolic activation of aromatic hydrocarbons have yet to be tested.

This report compares the carcinogenic activities of two bromomethylbenz[*a*]anthracenes with those of their parent hydrocarbons and is, therefore, of relevance to possible mechanisms of metabolic activation of methyl-substituted hydrocarbons which involve the methyl group as the critical site of metabolic attack [3, 9, 10]. It was accepted that such experiments represented, at best, only an artificial model of carcinogenesis by the parent hydrocarbons; the relative carcinogenic potencies of these reactive bromo-compounds were also considered, therefore, simply as a function of their own intrinsic chemical reactivities.

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### MATERIAL AND METHODS

*Syntheses*

Benz[*a*]anthracene (Koch-Light Laboratories, England) was routinely crystallized from acetone/water before use. 12-Methylbenz[*a*]-anthracene was prepared according to Cook *et al.* [11], and 7-methylbenz[*a*]anthracene by

the reduction of 7-chloromethylbenz[*a*]anthracene [12] with stannous chloride/HCl according to Wood and Fieser [13].

*7-Bromomethylbenz*[a]*anthracene* was obtained when 7-methylbenz[*a*]anthracene in carbon disulphide solution was cooled in a freezing mixture and treated dropwise with a molar equivalent of bromine. After 30 min the product in suspension was collected, washed with dry ether and recrystallized from benzene. 7-Bromomethylbenz[*a*]anthracene m.p. 190·5–191·5°C was obtained in 66% yield (found, C, 70·75; H, 4·35; Br, 24·85; $C_{19}H_{13}Br$ requires C, 71·05; H, 4·1; Br, 24·9%).

*7-Bromomethyl-12-methylbenz*[a]*anthracene* was obtained in 58% yield when 12-methylbenz[*a*]-anthracene (1 g) mixed with the clear solution obtained by passing hydrogen bromide through a suspension of paraformaldehyde (0·2 g) in glacial acetic acid (5 ml), was kept at room temperature overnight. After precipitation with water, drying and recrystallization from benzene–cyclohexane (1:1) the product was obtained as pale yellow needles, m.p. 150–152°C (found, C, 71·5; H, 5·05; Br, 23·9; $C_{20}H_{15}Br$ requires C, 71·65; H, 4·5; Br, 23·85%). After reduction with stannous chloride/HCl [13] this product yielded a compound indistinguishable from authentic 7,12-dimethylbenz[*a*]anthracene either by m.p. or chromatography in several solvent systems. Pataki *et al.* [14] have prepared 7-bromomethyl-12-methylbenz[*a*]anthracene by a different route and report its m.p. as 139–141°C.

7-Bromomethylbenz[*a*]anthracene, identical by m.p. and mixed m.p. with that described above, was also prepared by bromomethylation of benz[*a*]anthracene under similar conditions to those described for 7-bromomethyl-12-methylbenz[*a*]anthracene.

*Benz*[a]*anthranyl-7-methyl-*[4-(p-*nitrobenzyl*)]*pyridinium bromide* was prepared by adding 7-bromomethylbenz[*a*]anthracene (0·963 g) to a solution of 4-(*p*-nitrobenzyl)-pyridine (0·642 g) in dimethylsulphoxide (5 ml). As the bromide dissolved, the initial blue colour of the solution changed through green to yellow after one hour at room temperature. A yellow oil was then precipitated by ether, washed with ether and subsequently crystallized by warming with ethanol (1 ml). The crystals were slurried in acetone, collected, washed with acetone and recrystallized from methanol giving 1·17 g (73%) of benz[*a*]anthranyl-7-methyl-[4-(*p*-nitrobenzyl)] pyridinium bromide m.p., 223–225°C (found, C, 69·15; H, 4·3; N, 5·1; Br, 15·6; $C_{31}H_{23}BrN_2O_2$ requires C, 69·5; H, 4·35; N, 5·25; Br, 14·9%). This product, in ethanol which was 10 mM with respect to tri-isoamylamine, exhibited a peak at 544 nm (ε, 6400). When 0·3 ml benzene was added to 2 ml of this solution (i.e. conditions identical to those used for the kinetic studies) the peak absorbance remained at 544 nm but the molar extinction coefficient was raised to 7750.

*Benzyl-*[4-(p-*nitrobenzyl*)]*pyridinium bromide* was prepared in a similar fashion, but the reaction conditions were 17 hr at 37°C. The product crystallized from ethanol/methanol (2/1) as yellow crystals, m.p. 219–222°C (with decomposition) (found, C, 59·1; H, 4·55; N, 7·1; Br, 21·0; $C_{19}H_{17}BrN_2O_2$ requires C, 59·25; H, 4·45; N, 7·25; Br, 20·75%). This product, in ethanol, which was 10 mM with respect to tri-isoamylamine, exhibited a peak at 536 nm (ε, 4920). When 0·3 ml of benzene was added to 2 ml of this solution, the peak absorbance remained at 536 nm but the molar extinction coefficient was raised to 7710.

*Reactivity studies*

An aliquot (0·3 ml) of a 2 mM solution of the arylmethyl bromide in benzene was added to 2 ml of the nitrobenzylpyridine reagent [a solution in commercially supplied absolute ethanol, 80 mM with respect to 4-(*p*-nitrobenzyl)-pyridine and 10 mM with respect to tri-isoamylamine] maintained at 49·5°C in the heated cell housing of a Unicam S.P. 800 spectrophotometer. The absorption spectrum, measured against a reference solution containing no arylmethyl bromide, was recorded as a function of time.

*Carcinogenicity experiments*

Groups of ten CB-Hooded adult female rats were given a single subcutaneous injection in their right hind leg of 0·25 ml of a 1% solution of the test compound in dimethylsulphoxide (distilled at reduced pressure under nitrogen). There were no accidental deaths of animals without tumours, and all the tumours were classified by Dr. F. J. C. Roe of this Institute as spindle cell sarcoma. 7,12-Dimethylbenz[*a*]-anthracene was obtained commercially and 7-hydroxymethyl-12-methylbenz[*a*]anthracene was a gift from Dr. P. Sims.

## RESULTS

The results of the animal experiments are presented in Table 1. Under the conditions of this test, 7-bromomethyl-12-methylbenz[*a*]-anthracene was of comparable activity to its parent, 7,12-dimethylbenz[*a*]anthracene, and considerably more active than its hydrolysis product, 7-hydroxymethyl-12-methylbenz[*a*]-anthracene. 7-Bromomethylbenz[*a*]anthracene elicited no tumours in this test, and was less active, therefore, than its parent, 7-methylbenz[*a*]anthracene.

*Table* 1. *Tumour incidence in adult female rats*

| Compound (administered as 0·25 ml of a 1% solution in dimethylsulphoxide) | Cumulative number of rats (from groups of ten) with sarcoma at injection site | | |
|---|---|---|---|
| | 6 months | 9 months | 12 months |
| Vehicle (dimethylsulphoxide) | 0 | 0 | 0 |
| 7-Methylbenz[*a*]anthracene | 1 | 2 | 2 |
| 7-Bromomethylbenz[*a*]anthracene | 0 | 0 | 0 |
| 7,12-Dimethylbenz[*a*]anthracene | 8 | 9 | 9 |
| 7-Bromomethyl-12-methylbenz[*a*]anthracene | 6 | 9 | 10 |
| 7-Hydroxymethyl-12-methylbenz[*a*]anthracene | 0 | 0 | 0 |

The chemical reactivities of the arylmethyl bromides were compared by studying their reaction with 4-(*p*-nitrobenzyl)pyridine. The method was modified from that of Bardos *et al.* [15] by using this reagent in direct combination with tri-isoamylamine so that the colour development could be monitored directly. It was shown that under the conditions of these studies the extinction coefficient of the coloured product varied only slightly with changes in the aryl moiety of the arylmethyl bromide since there was less than 1% difference between the extinction coefficients of the benzyl- and benz[*a*]anthranyl-7-methyl derivatives (see Material and Methods).

It was also shown that the extinction coefficient of benzyl-[4-(*p*-nitrobenzyl])pyridinium bromide at its maximum, 536 nm, was independent of concentration in the concentration range used in the kinetic studies. It then follows from the known values of the extinction coefficients that if all the arylmethyl bromide reacted with the pyridine derivative, a final absorbance of 2·0 would result. Since this theoretical value was never achieved (see below) the products of the reaction, for the case of 7-bromomethylbenz[*a*]anthracene, were examined by thin layer chromatography on silica gel (Eastman Chromatogram Sheet 6060), in several solvent systems. In addition to benz[*a*]anthranyl-7-methyl-[4-(*p*-nitrobenzyl)]pyridinium bromide, only 7-hydroxymethylbenz[*a*]anthracene [12] was detected. 7-Ethoxymethylbenz[*a*]anthracene [12] was not

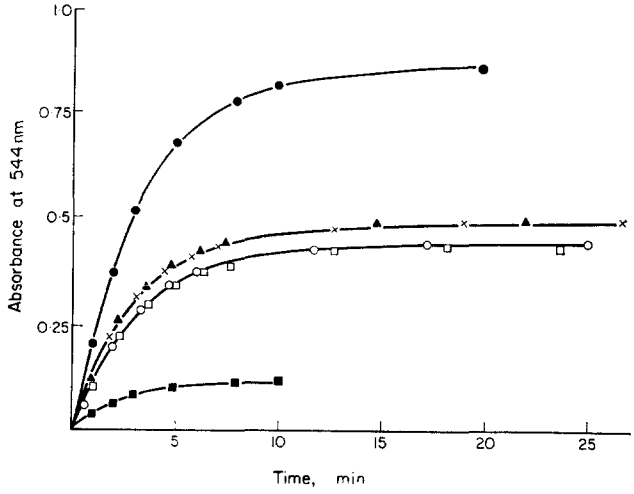


*Fig.* 1. *The effect of variations in water concentration and variations in* 4-(p-*nitrobenzyl)pyridine concentration on the reaction of* 7-*bromomethylbenz*[a]*anthracene with* 4-(p-*nitrobenzyl)-pyridine. The standard procedure described under Material and Methods was followed but the constitution of the standard nitrobenzylpyridine reagent was varied. The only deviation from the standard reagent for each case is indicated below:*

X—*standard reagent,* ▲—*the ethanol was dried over magnesium ethoxide,* O—*the ethanol was* 0·5 M *with respect to water,* □—*the ethanol was* 1 M *with respect to water,* ■—*the concentration of* 4-(p-*nitrobenzyl)pyridine was* 20 mM, ●—*the concentration of* 4-(p-*nitrobenzyl)pyridine was* 160 mM.

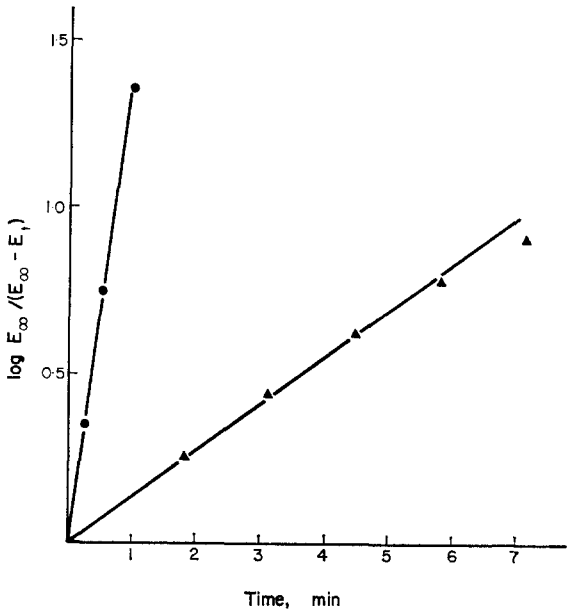


*Fig. 2. Analysis of the data from the reactions of 7-bromomethylbenz*[a]*anthracene (▲—▲) and 7-bromomethyl-12-methylbenz*[a]*anthracene (●—●) with 4-(p-nitrobenzyl)pyridine under the conditions described under Material and Methods.*

a product of the reaction. Therefore, in the kinetic studies two simultaneous reactions were occurring.

The effect of fluctuations in the water content of the solvent on the observed spectral changes was then examined for the case of 7-bromomethylbenz[*a*]anthracene (Fig. 1). It was seen that attempts to dry the ethanol with magnesium ethoxide caused no significant changes, and only slight variations were observed when the ethanol solution of the reagent was made either 0·5 M or 1 M with respect to water. The hydrolysis reaction was therefore reasonably assumed to be a first order reaction and it was further reasonable to assume that slight variation in the water content of the commercial absolute ethanol used for the standard reagent would not lead to significant changes in the data obtained. (This latter was also confirmed by experience in using the reagent made up from different batches of commercial alcohol). The reaction of 7-bromomethylbenz[*a*]anthracene with the pyridine reagent was, however, strikingly dependent on the concentration of the 4-(*p*-nitrobenzyl)pyridine (Fig. 1) and was therefore shown to be a second order reaction.

Such simultaneous reactions can be described as follows:

$$\text{RCH}_2\text{Br} \xrightarrow{k_1} \text{RCH}_2\text{OH}$$

$$\text{RCH}_2\text{Br} + p\text{NBP} \xrightarrow{k_2} \text{RCH}_2p\text{NBP}$$

where *p*NBP represents 4-(*p*-nitrobenzyl)pyridine. If $a$ denotes the initial concentration of $RCH_2Br$ and $x$ the amount reacted at time $t$, then, the rate of disappearance of $RCH_2Br$ is given by:

$$dx/dt = k_1(a - x) + k_2[p\text{NBP}]\ (a - x)$$
$$= (k_1 + k_2')\ (a - x)$$

where $k_2' = k_2[p\text{NBP}]$ since *p*NBP is present in sufficient excess for its concentration to be considered effectively constant.

The integrated form of this general pseudo first order rate equation is:

$$\ln a/(a - x) = kt$$
$$\text{where } k = k_1 + k_2'.$$

This can then be written in terms of the measured quantities:

$$\ln E_\infty/(E_\infty - E_t) = kt$$

where $E_\infty$ and $E_t$ are the absorbance at any given wavelength at infinite time (greater than five times the half life of the reaction) and at time, $t$, respectively.

The values of $k$ and its components $k_1$ and $k_2'$ can then be determined from the relationships:

$$\log E_\infty/(E_\infty - E_t) = kt/2{\cdot}303$$
$$k = k_1 + k_2'$$
$$\text{and} \qquad k_1/k_2' = (E_{\text{theor}} - E_\infty)/E_\infty$$

where $E_{\text{theor}}$ is the absorbance which would have been attained if all the arylmethyl bromide had reacted with the pyridine derivative. This

*Table 2. Comparison of chemical reactivities*

| Compound | $E_\infty$ | $t_{\frac{1}{2}}(min)$ | $k(min^{-1})$ | $k_1(min^{-1})$ | $k_2'(min^{-1})$ | $k_1/k_2'$ |
|---|---|---|---|---|---|---|
| 7-Bromomethyl-12-methylbenz[*a*]-anthracene | 0·185 | 0·2 | 3·2 | 2·9 | 0·3 | 9·7 |
| 7-Bromomethylbenz[*a*]-anthracene | 0·49 | 2·2 | 0·31 | 0·24 | 0·07 | 3·4 |

These data apply to the reactions of the bromo-compounds with 4-(*p*-nitrobenzyl)pyridine under the standard conditions defined in Material and Methods. The symbols are defined in the text.

value, as previously indicated, is known from the extinction coefficient of the coloured product. The experimental data for 7-bromomethylbenz[*a*]anthracene and 7-bromomethyl-12-methylbenz[*a*]anthracene are presented in Figure 2 and the derived values of $k$, $k_1$, $k_2'$ and the half-life, $t_{\frac{1}{2}}$, for these compounds are listed in Table 2.

The half-life of benzyl bromide under these conditions was considerably longer than that of the two benz[*a*]anthracene derivatives. It was not possible, therefore, to record a reliable value of $E_\infty$ for benzyl bromide since at long reaction times (several hours in this case) the rate of fading of the colour became significant. A maximum value of $k_1/k_2'$ of 0·075 can, however, be assigned to this compound from the data obtained. For the other compounds considered, where the half-lives were considerably shorter (Table 2), it was established that the value of $E_\infty$ was constant over more than five half-lives of their reactions.

## DISCUSSION

Under the conditions used for the carcinogenicity tests, 7-bromomethyl-12-methylbenz-[*a*]anthracene was a potent carcinogen whilst 7-bromomethylbenz[*a*]anthracene was inactive (Table 1). It is unlikely that the biological activity of the former compound resulted from its hydrolysis *in vivo* to 7-hydroxymethyl-12-methylbenz[*a*]anthracene, since this compound was itself inactive under the same conditions. Similarly, it can be argued that the lack of carcinogenicity found for 7-bromomethylbenz-[*a*]anthracene cannot be attributed to its deactivation by hydrolysis because this was the least reactive of the two bromo-compounds (Table 2).

The conditions used to study the reactivity of these bromo-compounds were selected for technical reasons and do not represent the conditions prevailing *in vivo*. However, it is likely that the ratios of these reactivities will be similar *in vivo*, to those determined in this model system. It follows from the observations summarized in Table 2 that the carcinogen, 7-bromomethyl-12-methylbenz[*a*]anthracene, has a half-life about ten times shorter than that of 7-bromomethylbenz[*a*]anthracene. Also, whilst the specific reaction rate for the second order component of the overall reaction, $k_2'$, is four and a half times greater for the carcinogen than for the non-carcinogen, the most notable difference lies in the specific reaction rate for the first order component of the reaction, $k_1$. The values of $k_1$ in Table 2 indicate that 7-bromomethyl-12-methylbenz-[*a*]anthracene undergoes a first order solvolysis about twelve times more readily than does 7-bromomethylbenz[*a*]anthracene.

Whilst this positive correlation between carcinogenic activity and ability to undergo a first order reaction may well be of significance it is clearly desirable to extend the range of compounds studied to test the generality of this relationship. Other workers have previously reported similar relationships, however. Bardos *et al.* [15] have shown that the tumour-inhibitory activities of a series of aromatic nitrogen mustards are more closely related to their first order solvolysis reactions than to their second order reactions, and ethyl methanesulphonate, which reacts to a greater extent through a first order process than does the methyl ester [16], is also more carcinogenic for rat kidney than the methyl ester [17].

The experimental data in this report can also be examined for relevance to the general mechanism of tumour initiation by methyl-substituted hydrocarbons. If aralkylating species, generated by metabolic attack at the methyl groups, were intermediates in the carcinogenic action of methylbenz[*a*]anthracenes [3, 10] then, at first sight, it might be expected that both of the tested bromo-compounds should have exhibited a greater carcinogenic activity than their respective parent hydrocarbons. This was not found experimentally (Table 1) and the present data

cannot, therefore, be construed as support for such a mechanism. However, the data does not completely exclude such a mechanism either, since it does not follow that the possible metabolites for which these bromo-compounds are models will exhibit chemical properties (e.g. the kinetic properties reported here) identical with those of these model compounds.

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## SUMMARY

*7-Bromomethylbenz*[a]*anthracene was less carcinogenic than the parent hydrocarbon, 7-methylbenz*[a]*anthracene, whereas 7-bromomethyl-12-methylbenz*[a]*anthracene was a potent carcinogen, giving* 100% *incidence of sarcoma in the rat after a single subcutaneous injection. The chemical reactivities of these two bromo-compounds were compared, and the more carcinogenic compound was shown to react more rapidly, and also to a greater extent through a first order process.*

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# The Production of Chronic Gastritis and Ulceration in the Glandular Stomach of Rats by Iodoacetamide (IAM)

ROSE YASIN and C. L. LEESE

*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London S.W.3*

## INTRODUCTION

A RELATIONSHIP between chronic gastritis, intestinal metaplasia and stomach cancer has been suggested by several workers [1–7]. Loss of gastric secretory function has been reported in patients manifesting such lesions [3, 8, 9]. It is therefore important to develop an experimental animal system in which both the pathogenesis of the non-malignant conditions and their role in gastric carcinogenesis could be evaluated. The rat was used for this experimental test system because of availability.

Spontaneous gastric lesions rarely occur in the rat. Tumours of the glandular stomach have been produced by long-term administration of a variety of carcinogens [10–15] but the incidence of adenocarcinoma analogous to the human disease was generally low. However, other lesions arising through abnormal regeneration of the glandular epithelium were frequently reported. Normal rats were used for the latter experiments. It is plausible that the production of lesions such as chronic gastritis in the glandular stomach of these animals prior to challenge with carcinogens might result in an increase in both the incidence and rate of development of adenocarcinoma of the glandular stomach.

While numerous chemicals (e.g. pilocarpine, reserpine, acetyl salicylic acid, corticoids) are capable of producing ulceration in the rat glandular stomach, these lesions are generally of the acute rather than the chronic type [16–20]. Moreover, prolonged exposure to these drugs frequently results in a high incidence of forestomach ulcers, perforations or profound emaciation of the animal. Lalich [21], however, reported the consistent production of chronic ulceration in the rat glandular stomach in the absence of these deleterious effects, by the continuous feeding of 0·1% iodoacetamide in the drinking water. This was the method adopted in the present study of the pathogenesis of gastritis and chronic ulceration. The present report describes (1) the sequence of histological lesions which develop during such treatment and (2) the effect of iodoacetamide on the gastric function.

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## MATERIAL AND METHODS

*Animals*

Male, Chester-Beatty-Wistar rats, of the same age were employed in this study. Separate groups of control and experimental animals were used for the histological (Group I) and the gastric (Group II) analyses. Group I consisted of 18 control and 36 experimental animals, and Group II of 46 controls and 44 experimental animals. The rats were fed a Purina Chow diet (Plowco Feeds Ltd.) containing 20% protein and drinking water *ad libitum*. At 5 weeks of age, iodoacetamide (0·1%) was added to the drinking water of the experimental rats

for a total of 25 weeks. The mortality rate resulting from the treatment was 8–10%, with most deaths occurring during the first few weeks. Contrary to Lalich [21] this compound did produce a toxic reaction in the rats, particularly during the first week of treatment, manifested by a mean weight loss of 24 g. Tolerance to the drug developed during the following weeks and these animals thereafter gained weight. Although the weight of the treated rats was consistently lower than the controls of the same age (Table 1), the weights of animals in both groups increased in a parallel manner during the course of treatment. One control and two experimental rats from Group I were sacrificed 1 week after the commencement of treatment and at various intervals thereafter, the stomachs and the proximal portion of the small intestine were immediately removed. In Group II, immediately following collection of the gastric juice by the Shay technique (see below), the stomach and a portion of the adjacent small intestine were removed from both control and treated animals, usually six of each.

*Table 1. Effect of iodoacetamide (IAM) feeding on body weight*

| Age weeks | Animals | IAM weeks | Body weight (g) |
|---|---|---|---|
| 6 | C(7) | | 230±39† |
| | T(6) | 1 | 121±15* |
| 7 | C(8) | | 306±33 |
| | T(7) | 2 | 139±8·5* |
| 8 | C(6) | | 360±42 |
| | T(6) | 3 | 217±29* |
| 9 | C(6) | | 391±16 |
| | T(6) | 4 | 255±33* |
| 12 | C(6) | | 532±22 |
| | T(6) | 7 | 336±52* |
| 15 | C(5) | | 535±67 |
| | T(6) | 10 | 355±45* |
| 20 | C(4) | | 616±35 |
| | T(6) | 15 | 393±52* |
| 25 | C(6) | | 706±42 |
| | T(4) | 20 | 472±76* |

Parentheses indicate number of rats used.
*$P$=0·01.
†±standard deviation.

*Preparation of the stomachs for microscopic analyses*

The stomachs and the attached portion of the small intestine were opened along the outer curvature, thoroughly washed out with 0·9% saline and pinned on a piece of cork, mucosa upwards. After macroscopic examination, the tissue was fixed for 48 hr in a solution containing 10% formalin and 0·9% saline for Group I and a solution containing 2% calcium acetate and 10% formalin for Group II, then cut into 6 longitudinal sections and embedded into a single paraffin block following dehydration. In this way the entire stomach including the small intestine was cut and mounted on a single slide. Two sections of the tissue were cut at three different levels in order to obtain as wide a scan of the tissues as possible, and one of each pair was stained with hematoxylin–eosin and Southgate's mucicarmine–hematoxylin for morphological examination.

*Collection of gastric juice*

The gastric juice was collected by means of the standard Shay pylorus ligation technique [22], following a 20–24-hr starvation period. The animals were fed 8% sucrose in the drinking water during the starvation in order to avoid an excessive decrease in their blood sugar levels. They were housed in individual cages and the animals were separated from their faeces by a wire grill to avoid coprophagy. After a 4-hr secretion period, the rats were anaesthetized with ether and the stomachs were removed. The gastric juice was collected by cutting a small hole in the forestomach and allowing the juice to drain into a beaker. The volume was then measured and the juice was stored on ice until analyzed for acid and pepsin.

*Gastric juice analyses*

The acid content was determined with the aid of the Radiometer automatic titrator, type TTT1A. Free acid was measured by titration to pH 3·5 with 0·01 N NaOH and the total acid by titration to pH 7·0. Pepsin was assayed by the Anson hemoglobin method [23] as described by Rick [24]. In this report one pepsin unit (PU) refers to the amount of enzyme which hydrolyzes 0·1 g of hemoglobin (pH 1·6) at 35·5°C in 10 min to produce that amount of trichloroacetic acid soluble products (in 5·0 ml) which gives the same optical density at 580 mμ (EEL 'Spectra' spectrophotometer) as 1 mmole of L-tyrosine, using the Folin Ciocalteu phenol reagent as the chromogenic agent.

Acid and pepsin were analyzed in terms of both concentration and output. Because of the relationship demonstrated between oxyntic and zymogenic cell mass to rat body weight [25] gastric juice volume, acid and pepsin output were calculated as the total secreted in 4 hr per 100 g of body weight.

## RESULTS

*Histological evaluation*

Iodoacetamide feeding consistently produced gastritis and chronic ulceration in the glandular

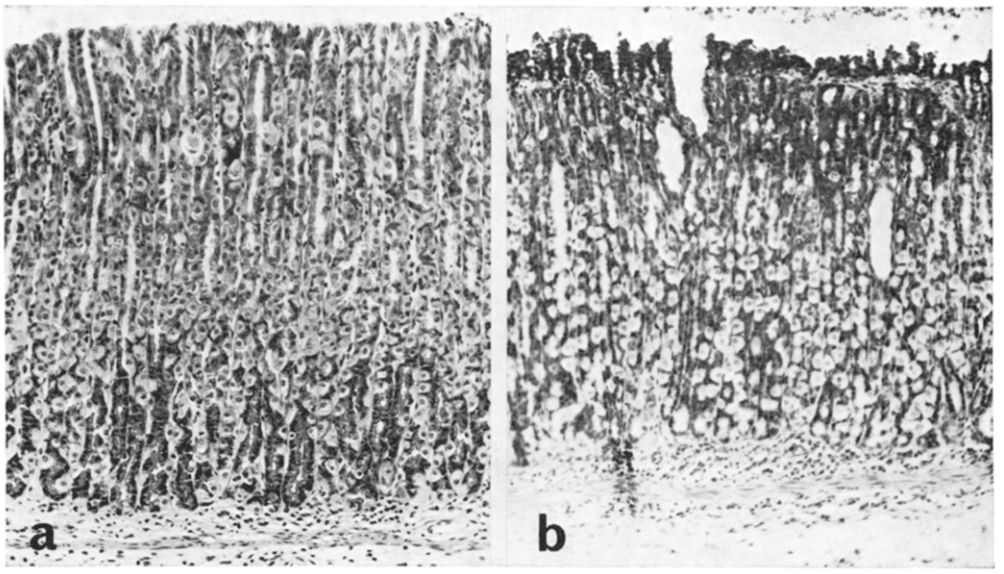


*Fig.* 1. *Normal and damaged body mucosa. Hematoxylin–eosin* × 160.
(a) *Sections of normal body mucosa taken from a* 6 *week old rat.*
(b) *Section of body mucosa taken from a* 7 *week old rat,* 2 *weeks after IAM treatment showing diffuse hyperemia and the abnormal superficial glands.*

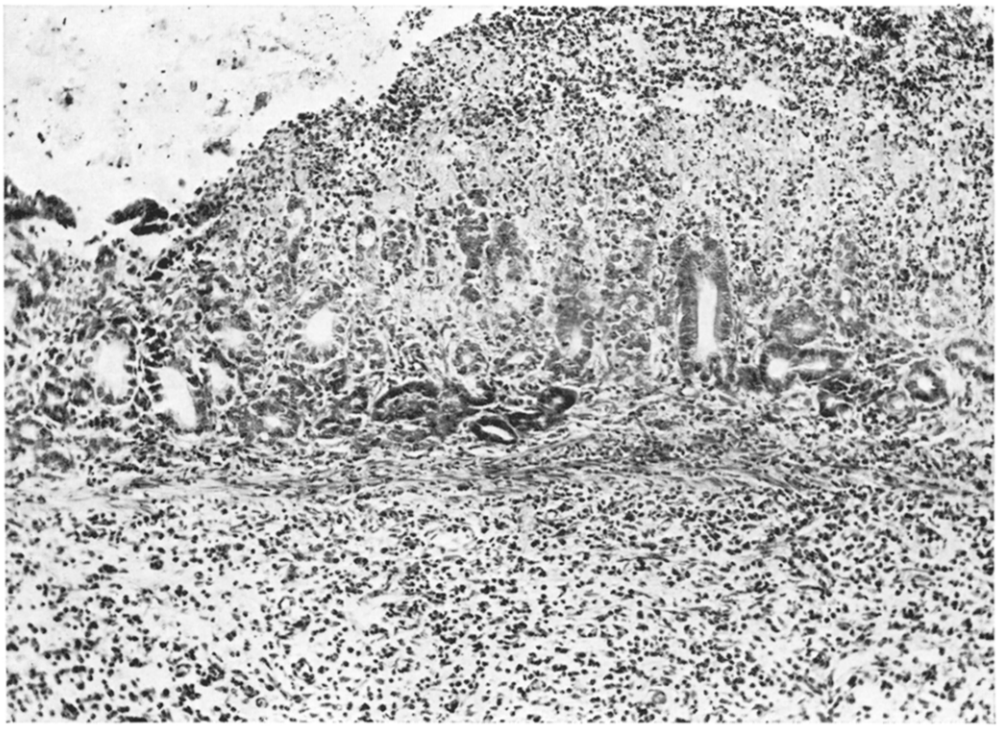

*Fig.* 2. *Section showing superficial ulceration of body mucosa taken from an* 8 *week old rat* 3 *weeks after IAM treatment. The surface of the ulcer is composed of a necrotic exudate overlying several basal glands. An acute inflammatory reaction is present throughout. Hematoxylin–eosin* × 160.

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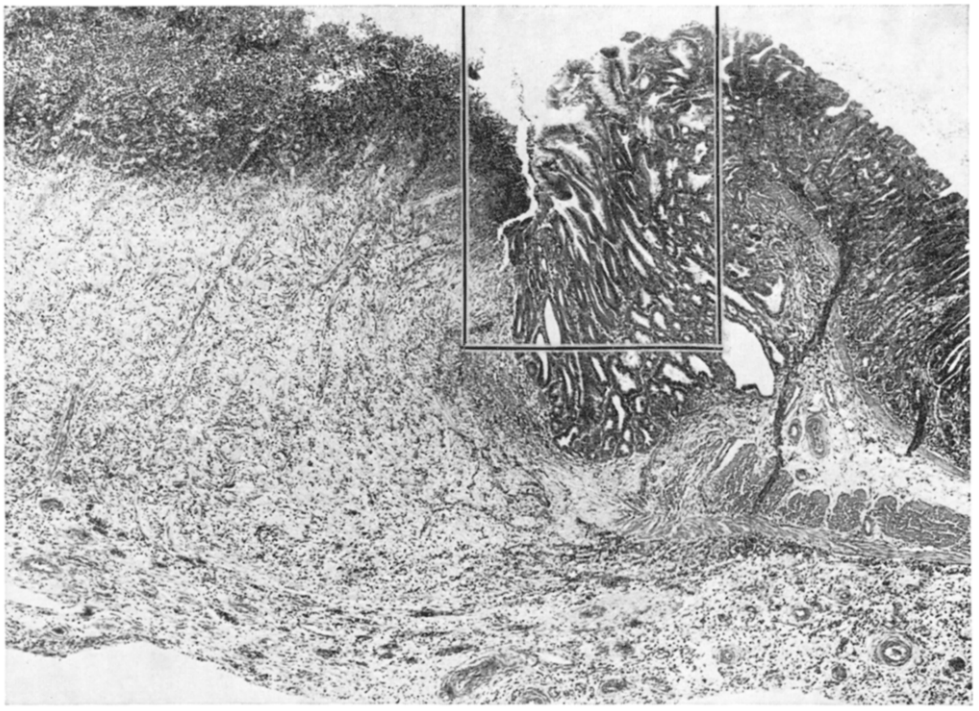

*Fig.* 3. *Section of a deep ulcer in the body region taken from a* 14 *week old rat,* 9 *weeks after IMA treatment. The ulcer has penetrated to the serosal lining. The deep glands lying adjacent to the ulcer are lined by atypical mucous cells. Hematoxylin–eosin* × 160.

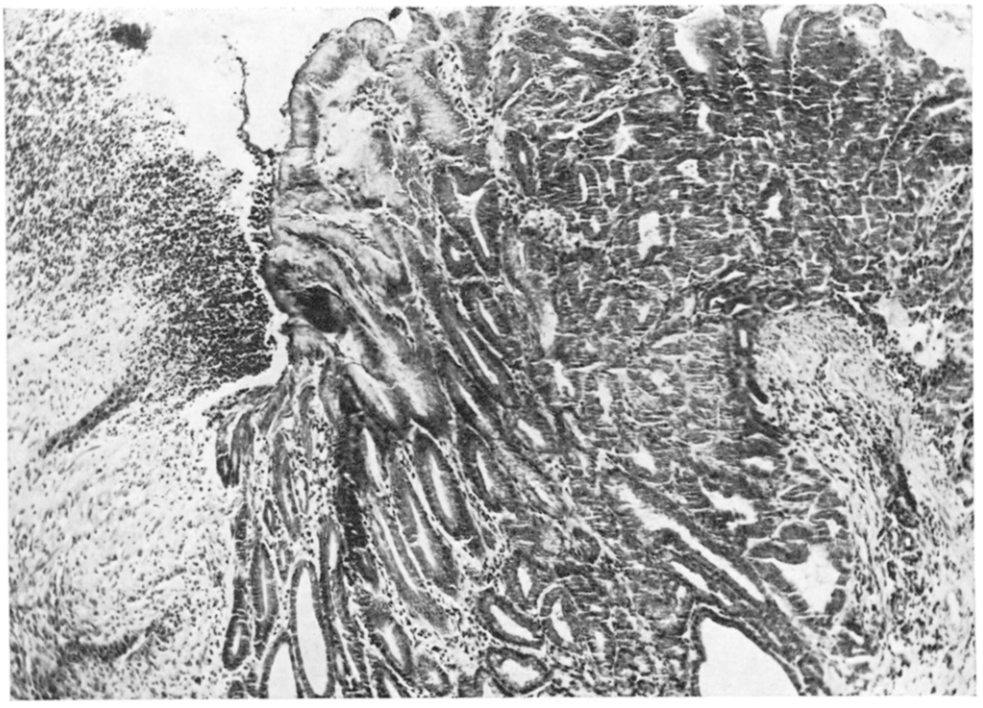

*Fig.* 4. *A higher magnification of the area outlined in Fig.* 3 *showing the atypical mucus secreting cells. Hematoxylin–mucicarmine* × 130.

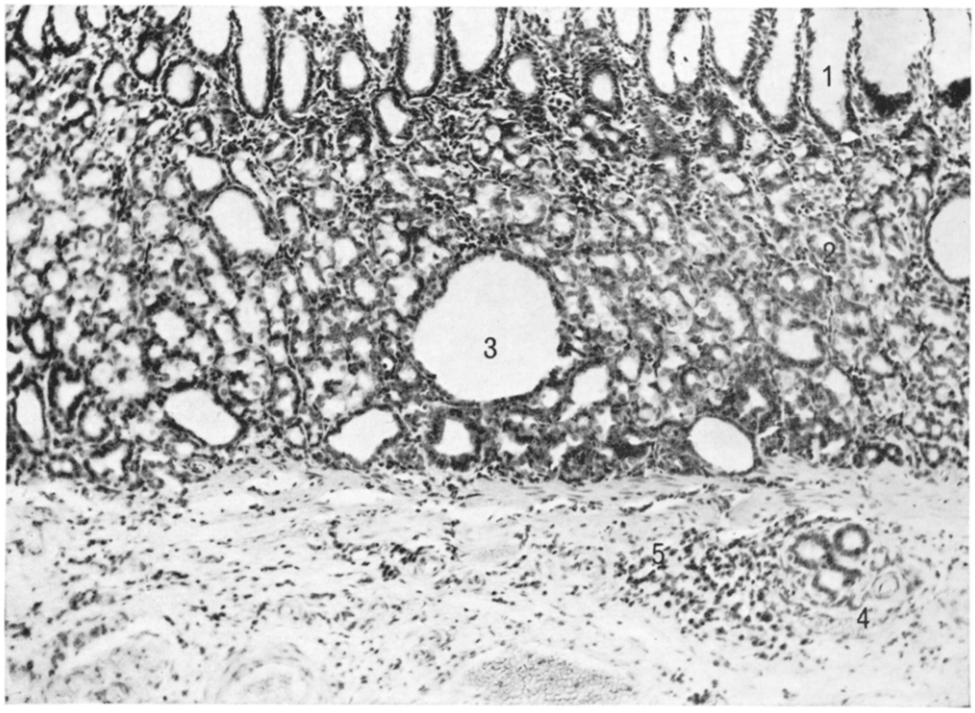


*Fig.* 5. *Section of atrophic body mucosa taken from a* 25 *week old rat,* 20 *weeks after IAM treatment showing:* 1. *lengthened superficial glands (see Fig.* 1*a for comparison with normal);* 2. *atypical mucus secreting glands;* 3. *dilated gland lined by cuboidal cells;* 4. *displaced gland in which mitotic figures are present;* 5. *aggregate of lymphocytes. Hematoxylin–eosin* × 160.

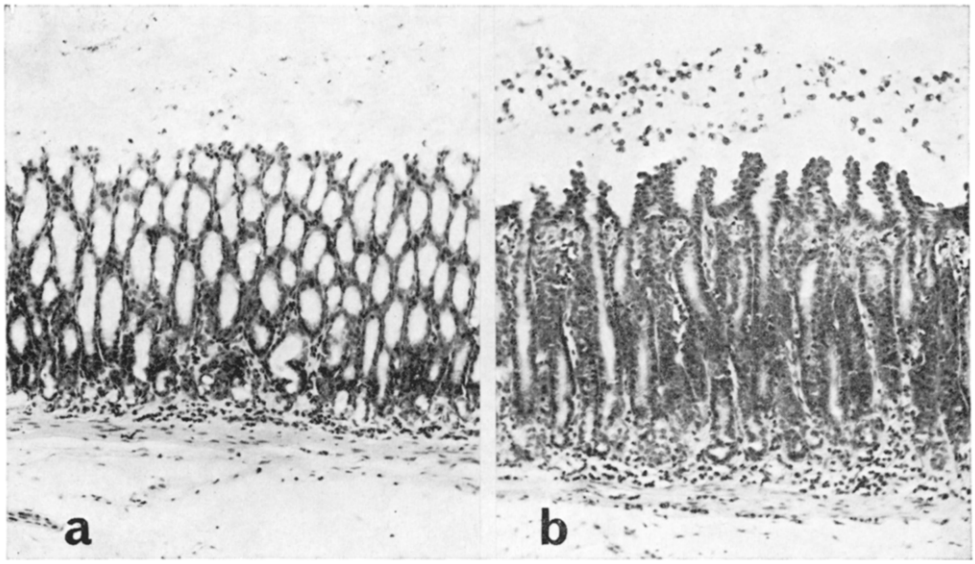


*Fig.* 6. *Normal and damaged pylorus. Hematoxylin–eosin* × 160.

(a) *Section of normal pylorus taken from a* 25 *week old rat.*

(b) *Section of pylorus taken from a* 20 *week old rat after 15 weeks of IAM treatment showing elongated superficial glands extruding large numbers of cells.*

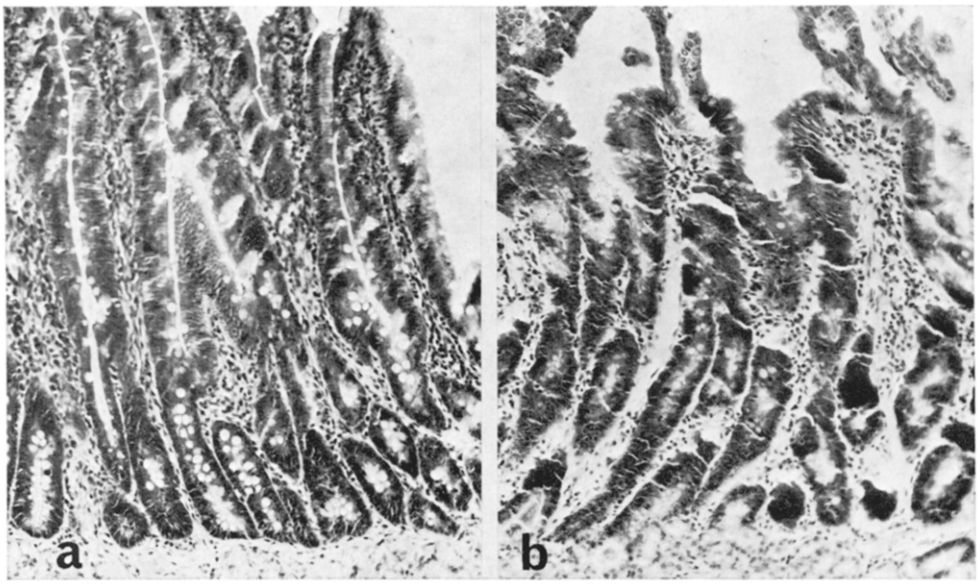


*Fig.* 7. *Normal and damaged duodenum. Hematoxylin–eosin* × 160.
(a) *Section of normal duodenal epithelium taken from an* 8 *week old rat.*
(b) *Section of abnormal duodenal epithelium* (*same age as* (a)) *taken* 3 *weeks after IAM treatment showing shortened villi with finger-like projections and elongated crypts.*

stomach of the rats. Forestomach ulcers were occasionally observed but only during the last weeks of treatment. The lesions were more frequently located on the dorsal side of the outer curvature and included both the cardiac and body regions.

The initial effect of iodoacetamide on the body mucosa was the production of diffuse hyperemia beneath the tips of the superficial glands and focal necrosis principally in the proximal portion of the body epithelium. This effect was observed after the first week of treatment. The surface glands overlying the hyperemic zones were either flattened or elongated (Fig. 1b) and appeared to be extruding a greater number of cells into the lumen of the stomach than did the controls (Fig. 1a). The next stage in the ulcerogenic process was the development of superficial ulcers involving the mucosal epithelium but only of the proximal body region (Fig. 2). The surface of the ulcers consisted of inflammatory and necrotic exudate but some basal glands remained visible. These lesions were accompanied by an acute inflammatory reaction with leucocytes and eosinophils being the most prominent cells. With continued treatment, the ulcers increased in size and depth eventually penetrating through the muscularis mucosae. During the final stages of the ulcerogenic process, the lesions were often extensive, involving a large area of the dorsal body mucosa and to a lesser extent the ventral outer curvature with the consequent loss of specialized secreting tissue. These ulcers now penetrated deep into the musculature and frequently extended to the serosal lining (Fig. 3). In some cases ulceration penetrated the serosal lining thus resulting in 'confined perforations' where contiguous structure adhered to the ulcerated portion of the stomach wall. The glands lying adjacent to such ulcers consisted of long deep glands lined by atypical mucous secreting cells which ranged from tall columnar to cuboidal in shape (Fig. 4). The muscularis mucosae was absent in this region and the glands frequently penetrated into the submucosa, some of them being dilated and lined with cuboidal cells. This atypical mucous cell metaplasia may represent a process similar to the intestinal metaplasia seen in diseased human stomach although the cells in this case differ in structure from the goblet cells of the rat small intestine.

Localized zones of atrophic body were encountered from the fourth week of treatment onwards, presumably the result of abnormal repair of the damaged areas. The pattern of regeneration and atrophy appeared to be related to the state of the muscularis mucosae which in turn depended on the depth of previous ulceration. When the muscularis mucosae remained intact, the glands repaired in a relatively normal manner although glands lined by atypical mucous cells and a thinning of the epithelium were frequently encountered. An increased number of lymphocytes in the submucosa was often observed beneath these defects. The fracturing or loss of the muscularis mucosae resulted in a variety of lesions with the following characteristics: (1) disorganization and thinning of the mucosal epithelium, (2) a loss of the zymogenic and oxyntic cells with a corresponding increase in mucus secreting cells; (3) the presence of dilated glands lined by cuboidal cells which frequently grew downward into the submucosa; (4) nests of epithelial cells in the submucosa beneath a fragmented muscularis mucosae; (5) the presence of atypical mucous cells; (6) the presence of a chronic inflammatory reaction, the predominant cells being lymphocytes. Such lesions resemble, in many respects, the atrophic gastritic condition present in human stomach and which often accompany gastric ulcers and cancer.

Figure 5 illustrates some of these forms of atrophy but the degree is only moderate. The main features of this reaction are (1) the increased length of the superficial glands; (2) localized glands with atypical mucous cells in regions normally inhabited by secretory cells; (3) dilated glands lined by cuboidal cells; (4) a displaced nest of epithelial cells in the submucosa manifesting mitotic activity; (5) a chronic inflammatory action indicated by the aggregate of lymphocytes in the submucosa adjacent to the displaced nest of epithelial cells.

The pylorus appeared to be generally more resistant to ulceration than the body epithelium. Superficial ulcers were observed in the prepyloric zones during the latter half of the treatment, involving the mucosal epithelium only. Abnormal repair and glandular atrophy ensued and exhibited a pattern similar to that described for the body region. The most characteristic abnormality observed in the pylorus was the structural malformation of the superficial epithelium which appeared after the first week of iodoacetamide feeding, and persisted with varying intensity throughout the course of treatment. Figure 6b illustrates this lesion. The tips of the glands were elongated extruding large numbers of cells into the lumen of the stomach. An increased mitotic activity accompanied this abnormality (not visible at the illustrated magnification) but unlike normal

*Table 2. Effect of iodoacetamide (IAM) feeding on acid secretion*

| Age wk | | IAM wk | H+ concentration§ Free | H+ concentration§ Total | H+ output¶ Free | H+ output¶ Total |
|---|---|---|---|---|---|---|
| 6 | C(7) | | 68±10‡ | 85± 9‡ | 193± 52‡ | 239± 55‡ |
| | T(6) | 1 | 29±10* | 37±10* | 277±145 | 349±159 |
| 7 | C(8) | | 47±20 | 75±17 | 75± 75 | 107± 82 |
| | T(7) | | 39±20 | 52±20 | 313±147* | 414±155* |
| 8 | C(6) | 2 | 51±23 | 77±19 | 77± 56 | 110± 62 |
| | T(6) | 3 | 49±16 | 67±15 | 321±142* | 427±132* |
| 9 | C(6) | | 53±18 | 85±16 | 64± 47 | 98± 62 |
| | T(6) | 4 | 51±16 | 63±14 | 309±195† | 373±222† |
| 12 | C(6) | | 40±13 | 70±14 | 48± 24 | 86± 29 |
| | T(6) | 7 | 37±12 | 51±10† | 172± 30† | 229±101† |
| 15 | C(5) | | 40±10 | 69±15 | 31± 14 | 53± 21 |
| | T(6) | 10 | 38±10 | 53±10† | 125± 48* | 174± 62* |
| 20 | C(4) | | 32± 7 | 59±10 | 33± 32 | 53± 42 |
| | T(6) | 13 | 23± 4 | 43± 8† | 79± 34† | 150± 69† |
| 25 | C(6) | | 12±12 | 46± 9 | 8± 11 | 29± 14 |
| | T(4) | 20 | 25± 4† | 45± 4 | 52± 19* | 92± 28* |

Parentheses indicate number of rats used.
*$P=0{\cdot}01$.
†$P=0{\cdot}05$.
‡±standard deviation.
§$H^+$ Concentration—mEq/l.
¶$H^+$ Output—μEq/4 hr per 100 g body weight.

pyloric glands where mitosis occurs principally in the crypts, the mitotic figures were observed at higher levels in the affected glands. Following the first week of treatment a similar abnormality developed in the duodenum and proximal jejunum (Fig. 7b). In this case the crypts were elongated and the villi shortened. The villi were no longer rounded as in normal small intestine (Fig. 7a) but contained finger-like projections from which rows of cells were being extruded. Similar to the pylorus, an increased mitotic activity was present extending nearly the full length of these elongated crypts. Such alterations in the glandular structure of these two tissues suggest that some modification in the cell proliferation kinetics has occurred as a result of iodoacetamide feeding.

*Gastric function*

Table 2 summarizes the results obtained for the gastric secretion patterns of the control and treated rats.

(a) *Volume output* (*Fig.* 8). Iodoacetamide feeding resulted in a significant increase in the total volume of gastric juice secreted during the 4 hr pylorus ligation period throughout the course of treatment. The increase was most striking in the first four weeks of treatment but thereafter progressively decreased with time. After 20 weeks of iodoacetamide the experi-

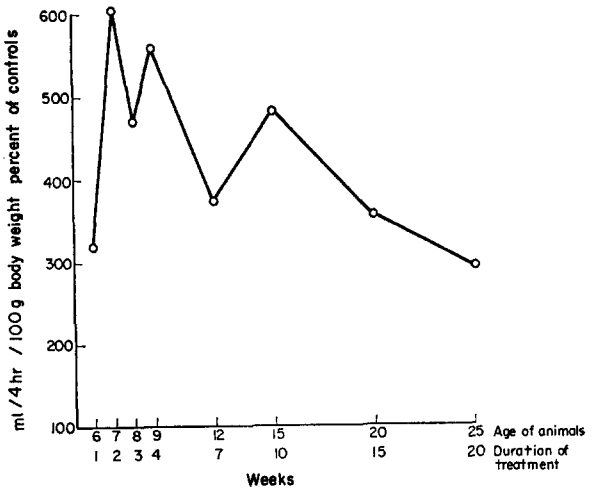


*Fig.* 8. *Effect of IAM treatment on the volume output. All points represent mean values.*

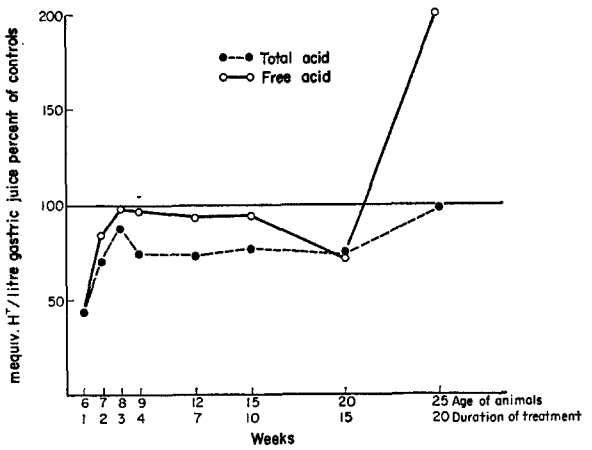


*Fig.* 9. *Effects of IAM treatment on the free and total acid concentration of gastric juice. All points represent mean value.*

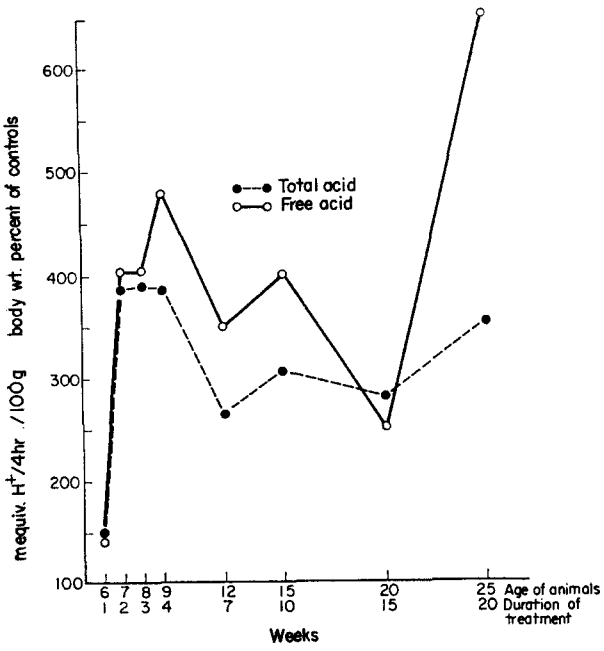


*Fig.* 10. *Effect of IAM treatment on the free and total acid output. All points represent mean values.*

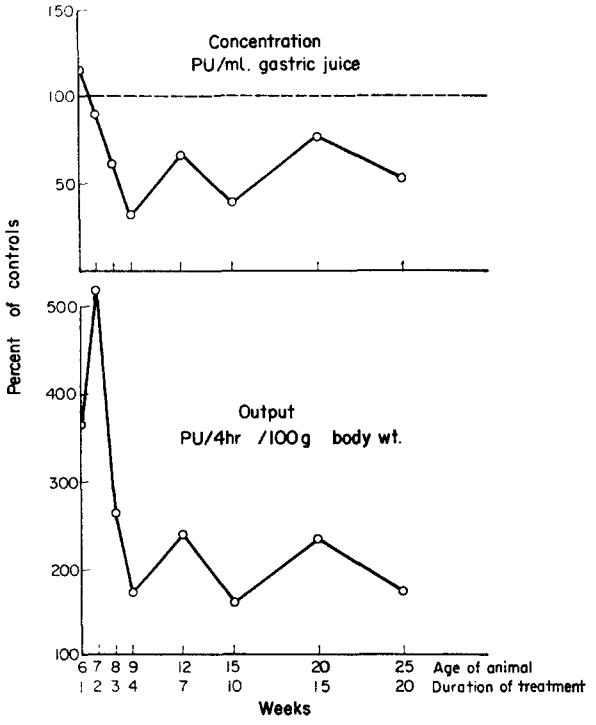


*Fig.* 11. *Effect of IAM treatment on the pepsin concentration of gastric juice and the total pepsin output. All points represent mean values.*

mental group continued to exhibit a volume output 2½ times greater than the normal control animals.

(b) *Acid concentration and output* (*Figs.* 9 *and* 10). A 55% decrease was observed in the mean free and total acid *concentration* following one week of iodoacetamide feeding. After the second week of treatment the acid concentration approached those obtained for the controls of the same age and continued to follow the normal pattern until 20 weeks of age (15 weeks iodoacetamide). However, at 25 weeks (20 weeks iodoacetamide) the free acid *concentration* in the treated group exceeded the control value by 200% despite the considerable loss of acid secreting cells as a result of extensive ulceration. In contrast to the acid *concentration* effect, an increase in free and total acid output was observed after 2 weeks of iodoacetamide administration which persisted throughout most of the experimental period (Fig. 10).

(c) *Pepsin concentration and output* (*Fig.* 11). Iodoacetamide affected the pepsin *concentration* of the gastric juice in a somewhat different manner to that observed for the acid *concentration*. No alteration in the pepsin *concentration* was observed during the first two weeks of treatment. In the four subsequent weeks the *concentration* decreased significantly but returned thereafter to the normal control values for the remainder of the treatment. Pepsin *output* manifested a reversed pattern. A marked increase was observed during the first 2 weeks of treatment (Fig. 11) which declined rapidly during the following 5 weeks towards the normal control values. However, a significant rise in pepsin *output* similar to that obtained for the acid *output* was again observed during the final week of the experimental period.

## DISCUSSION

Lalich [2] reported the consistent production of chronic ulceration in the dorsal epithelium of the rat glandular stomach, beneath the forestomach ridge, following 10 weeks continuous feeding of aqueous iodoacetamide. The results obtained in this investigation confirm these findings. However, contrary to his report of the absence of abnormalities in other regions of the gastro-intestinal tract, in this study lesions were also observed in the pylorus and upper small intestine which indicated an imbalance between cell loss and cell proliferation. Accompanying this induced gastritic condition was the enhancement in the volume, acid and pepsin *output* of the remaining intact secretory tissue. Several workers have shown that a 1 mM concentration of iodoacetamide completely inhibits acid secretion by frog and mouse mucosa, under *in vitro* conditions [26]. A 5·4 mM concentration was used in this study but the acid secretory mechanism was not thus affected:

one possibility is that the effective concentration of this compound was lower due to dilution in the stomach. In addition, the different secretion patterns observed for acid and pepsin suggests that there may be an independent response by the oxyntic and zymogenic cells to iodoacetamide feeding.

A similar stimulation in gastric secretion was reported during pilocarpine damage to rat gastric mucosa [19]. It is interesting that the damage resulting from this compound also occurred in the proximal body epithelium.

Despite the loss of secretory tissue during the final weeks of treatment — as a result of extensive ulceration — the enhanced values obtained for volume, acid and pepsin *outputs* suggests that the remaining secretory cells were functioning at an increased rate. On the basis of the histological lesions observed in this study, two possible mechanisms for this increase are proposed, both of which may be operating. The first could involve the release of histamine by back diffusion of acid into the damaged tissue, thereby stimulating acid secretion and increasing mucosal capillary permeability with the latter resulting in leakage of interstitial fluid into the lumen of the stomach; this scheme was proposed by Davenport [27] for acetic and salicylic acid damage to dog gastric mucosa. The second possibility is that the alteration in the cell proliferation kinetics of both the pylorus and upper small intestine results in some imbalance in the production of humoral agents (which normally control gastric function [28, 29]) by these tissues.

The mechanism by which iodoacetamide feeding produces chronic gastritis and ulceration in the glandular stomach of the rat remains unclear. Nevertheless, on the basis of the present findings, a tentative mechanism can be suggested. The earliest lesions observed as a result of the iodoacetamide treatment were hyperemia, abnormal surface cells and focal necrosis. These conditions were most severe in the proximal body epithelium and the prepyloric zone, regions which are probably exposed to higher concentrations of the compound. Thus damage to the surface cells and blood vessels may be directly initiated by iodoacetamide which is known to inhibit glycolysis and cell function. The shedding of dead surface cells which follows would result in the disruption of the protective mucosal barrier, rendering the epithelium more permeable to water soluble compounds and small ions and eventually leading to peptic digestion. While epithelialization of the damaged areas does ensue, the resulting structures were frequently abnormal and thus more susceptible to proteolytic damage. This sequence of recurrent ulceration would result in a chronic condition.

Contrary to the impressions conveyed by Lalich's report [21], lesions were also observed in the proximal small intestine. In addition the

*Table 3. Effect of iodoacetamide (IAM) feeding on gastric volume output and pepsin secretion*

| Age | Animals | IAM | Volume§ output | Pepsin¶ concentration | Pepsin ‖ output |
|---|---|---|---|---|---|
| 6 | C(7) | | 2·8±0·5‡ | 32±11‡ | 94± 26‡ |
| | T(6) | 1 | 9·1±2·4* | 37± 8 | 347± 37* |
| 7 | C(8) | | 1·3±0·7 | 44±18 | 61± 42 |
| | T(7) | 2 | 7·9±1·6* | 39±11 | 314±117* |
| 8 | C(6) | | 1·4±0·6 | 43±17 | 63± 38 |
| | T(6) | 3 | 6·4±1·4* | 26± 7† | 168± 72* |
| 9 | C(6) | | 1·0±0·4 | 66±15 | 75± 44 |
| | T(6) | 4 | 5·6±2·7* | 21± 9* | 129±101 |
| 12 | C(6) | | 1·2±0·2 | 48± 7 | 56± 18 |
| | T(6) | 7 | 4·3±1·2* | 32±11 | 137± 50† |
| 15 | C(5) | | 0·8±0·2 | 58±28 | 45± 28 |
| | T(6) | 10 | 3·7±1·0* | 22± 9† | 70± 39 |
| 20 | C(4) | | 0·9±0·7 | 38±12 | 41± 45 |
| | T(6) | 15 | 3·4±1·2* | 29±10 | 97± 48 |
| 25 | C(6) | | 0·7±0·3 | 41±25 | 25± 16 |
| | T(4) | 20 | 2·0±0·5* | 21± 5 | 43± 16† |

Parentheses indicate number of rats used.
*$P$=0·01.
†$P$=0·05.
‡±standard deviation.
§Volume output—ml gastric juice secreted/4 hr per 10 g body weight.
¶Pepsin concentration—PU/ml gastric juice.
‖Pepsin output—PU/4 hr per 100 g body weight.

treated animals consistently showed a lower body weight compared with the controls, during the course of treatment. The latter could be due to a nutritional deficiency resulting from the malfunctioning of the absorptive cells in the damaged intestinal villi and/or a reduction in food intake. Such events may exacerbate the direct effect of iodoacetamide observed in this study. Several workers have reported lesions in the glandular stomachs of rats as a result of nutritional deficiencies [30, 31]. Furthermore an increase in the incidence of atrophic gastritis has been reported in patients with tropical sprue [32], a condition where the intestinal villi are shortened or absent and the crypts elongated. It would therefore be important in future studies of this type to design experiments which would measure and eliminate the contribution of such nutritional deficiencies, if present, to gastric mucosal integrity and gastric secretion.

The gastritic lesions observed in this study have many similarities to those seen in the corresponding human disease. Several workers have suggested chronic gastritis and intestinal metaplasia as precursors of gastric carcinoma [1–3]. This does not necessarily mean that this is an obligatory process but rather a statistical probability. Whether the presence of these induced lesions in the rat stomach will enhance the development of gastric carcinoma is yet to be determined. We have made some attempts to evaluate this possibility by challenging the stomach of rats with carcinogens such as urethane and N-methyl-N'-nitro-nitrosoguanidine, following one month pretreatment with iodoacetamide. So far we have not been successful in developing carcinoma (using serosal invasion as a necessary criteria) although we have observed the downgrowth of epithelial elements into the musculature. The main effect of these carcinogens was an accentuation of the atrophic lesions described above, combined with a reduction in their time of development. The failure to induce invasive adenocarcinoma may be due to two factors: (1) the time at which the carcinogens are introduced after the initiation of gastritis and (2) the latent period necessary for the development of such malignancies.

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## SUMMARY

*Iodoacetamide consistently produced chronic gastritis and ulceration, the initial effect being the production of diffuse hyperemia, focal necrosis and other abnormalities in the mucosal epithelium. With continued treatment ulceration of the body mucosa became extensive and chronic gastritis ensued. Pyloric and duodenal epithelium exhibited disturbances in proliferation kinetics.*

*Gastric juice volume, acid and pepsin output were consistently increased in treated animals suggesting that the rate of secretion is not inhibited by iodoacetamide despite the presence of extensive ulceration but that modification of some normal control mechanisms occurs. Ulceration and gastritis is considered to arise through damage to the mucosal barrier by iodoacetamide thereby lowering the resistance of the mucosal epithelium to proteolysis; recurrent damage producing chronic gastritis.*

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# The Carcinogenic Activity of Dibutylnitrosamine in IF×C57 Mice

M. WOOD, A. FLAKS and D. B. CLAYSON

*The Department of Experimental Pathology and Cancer Research, The School of Medicine, Leeds LS2 9NL, England*

## INTRODUCTION

DIBUTYLNITROSAMINE (DBNA) is a potent bladder carcinogen in the rat [1, 2] and guinea pig [3]. It and 4′-hydroxybutyl-butylnitrosamine are unique among the dialkylnitrosamines in their ability to induce bladder tumours [4]. In this paper DBNA is shown to be a bladder carcinogen in mice.

DBNA and the three potent bladder carcinogens in mice that were already known, namely 4-ethylsulphonylnaphthalene-1-sulphonamide (ENS) [5, 6, 7] and the two aromatic amines, 2-acetylaminofluorene [8, 9, 10, 11] and 2-aminodiphenylene oxide [12], all induced hyperplasia of the bladder epithelium after oral administration for 2–12 weeks [13]. ENS was exceptional in the magnitude of the proliferative response it induced in the bladder epithelium after one dose [14, 15]. The importance of this response in the ultimate development of bladder tumours is not clear but would be easier to assess if the acute response to further murine bladder carcinogens were known. Therefore the effect of a single dose of DBNA on the bladder epithelium has been investigated.

No bladder tumours have so far been reported to result from the treatment of neonatal mice with a limited amount of a chemical carcinogen [16]. This may be because of the special properties of the bladder epithelium or the choice of unsuitable test chemicals. In particular, it was apparent that no chemical which was known to induce bladder tumours in adult mice has been adequately tested in neonatal animals and it was decided to investigate DBNA. It was considered possible that DBNA might be an incomplete carcinogen in neonatal mouse bladder and therefore further mice were set up in which treatment with DBNA when young was followed by the implantation of a paraffin wax pellet into the lumen of the bladder.

A preliminary report on these results has appeared [17]. While these experiments were in progress, Takayama and Imaizumi [18] reported liver and oesophageal tumours in mice after feeding DBNA in the diet and Bertram and Craig [19] found both oesophageal and bladder tumours after giving the chemical in the drinking water.

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## MATERIAL AND METHODS

IF×C57 F1 hybrid mice were bred in the laboratory and maintained on Oxo 41B diet and water *ad libitum*. IF mothers of IF×C57 litters were fed the same diet during pregnancy and weaning of the neonatally-treated animals.

Dibutylnitrosamine (DBNA) was synthesized by Mr. R. Turner, from dibutylamine (Koch Light, Colnbrook, England). It travelled as one peak on gas chromatography (Perkin Elmer F11: Apiezon L, and Carbowax 1540 and KOH, coated capillary columns) and was judged pure. 2-Acetylaminofluorene was obtained from Koch Light Ltd. (Colnbrook, England) and [$^3$H]-thymidine (5 mCi/mmole) from the Radiochemical Centre (Amersham, England).

DBNA (10μl) was injected subcutaneously into the flank of 12-week old mice by Alga micropipette. In the long-term experiments it was given every 2 weeks for 40 weeks. To pro-

*Table* 1. *The carcinogenic activity of DBNA and 2-acetylaminofluorene in IF×C57 mice*

| Treatment | No. and sex | Mean survival (weeks) | Normal | Bladder epithelium* | | | | | | | Liver* | | Other |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Hyperplasia | | | Papilloma | Carcinoma | | | Adenoma | Hepatoma | |
| | | | | slight | moderate | severe | | I | II | III | | | |
| DBNA (10 µl, 2 weekly) | 17 F | 54 | 0 | 1 | 1 | 8 | 0 | 5 | 1 | 1 | 0 | 1 | 2† |
| | 12 M | 46 | 0 | 0 | 1 | 4 | 1 | 3 | 1 | 2 | 0 | 0 | 1† |
| 2-AAF (0·05% in diet) | 34 F | 64 | 5 | 14 | 11 | 2 | 1 | 1 | 0 | 0 | 1 | 32 | |
| | 20 M | 64 | 0 | 3 | 4 | 2 | 4 | 5 | 0 | 2 | 1 | 6 | 1‡ |
| None | 17 F | 68 | 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | 13 M | 68 | 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | |

*Most advanced lesion only is listed for each animal.
†Lung adenoma.
‡Sebaceous gland adenoma near the anus.

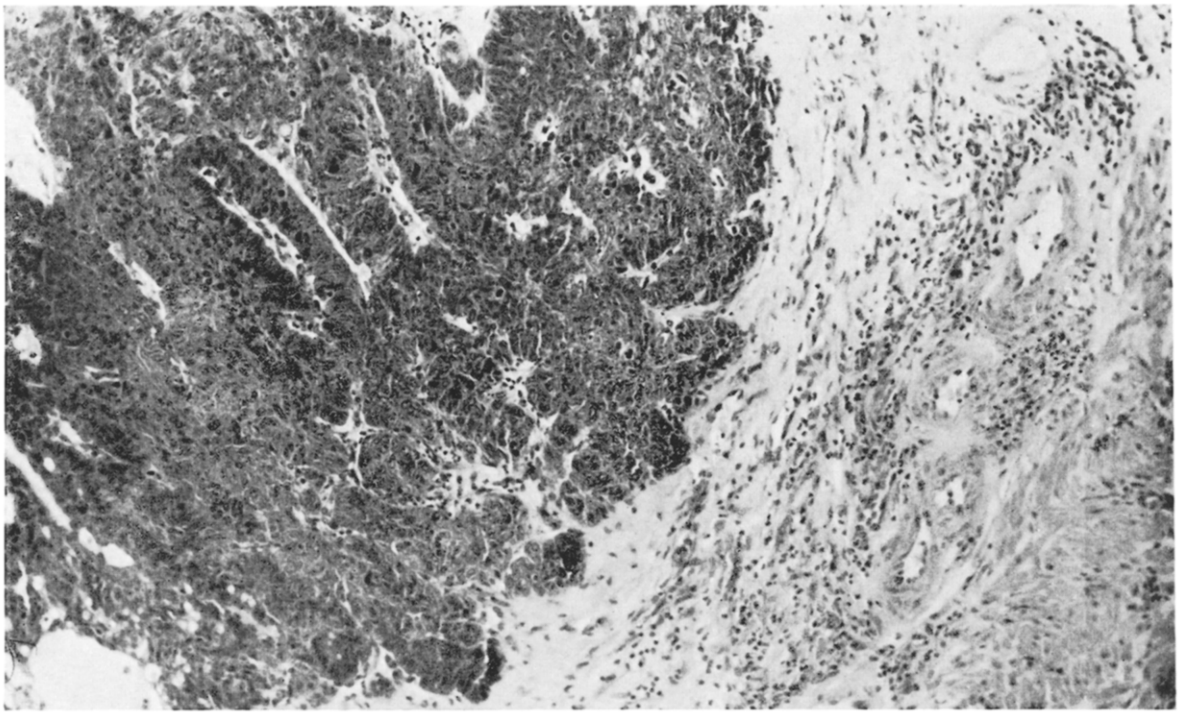

*Fig.* 1. (×63) *Area from the base of a transitional cell papilloma.*

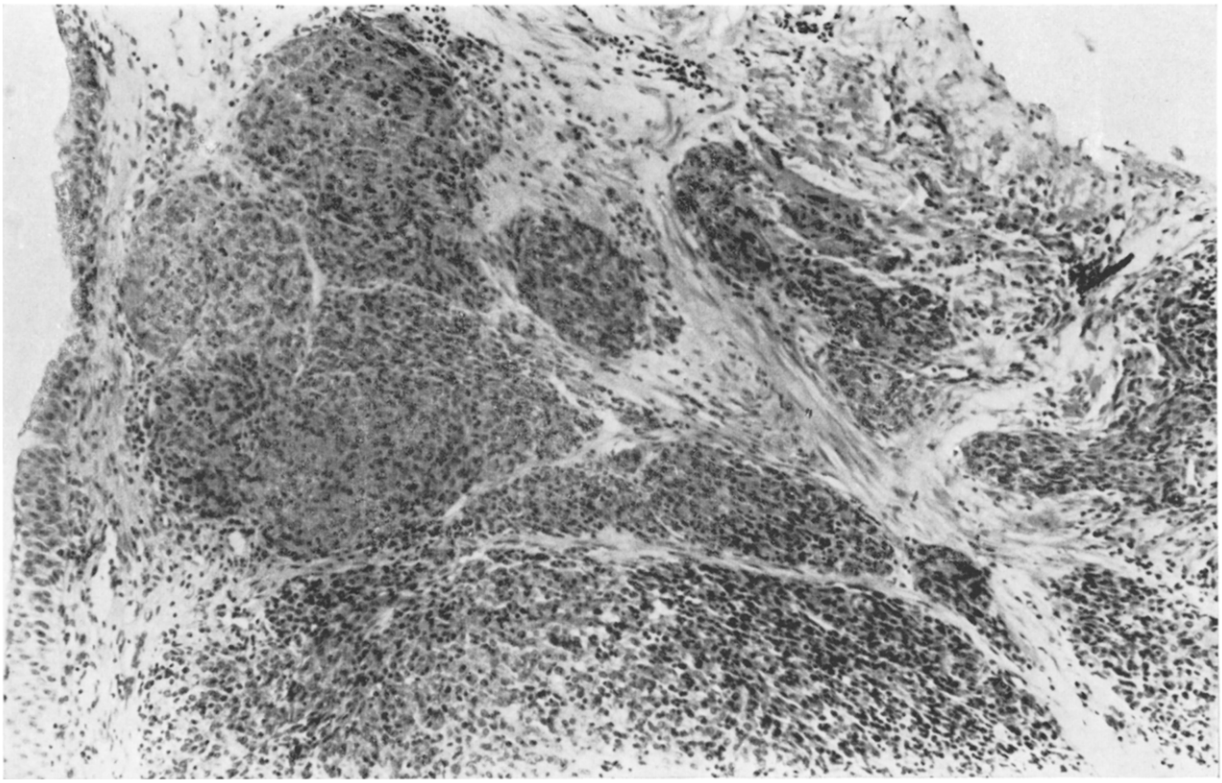

*Fig.* 2. (×63) *Solid islands of anaplastic carcinoma invading the bladder wall with hyperplastic epithelium and bladder lumen on the left and muscle on the right.*

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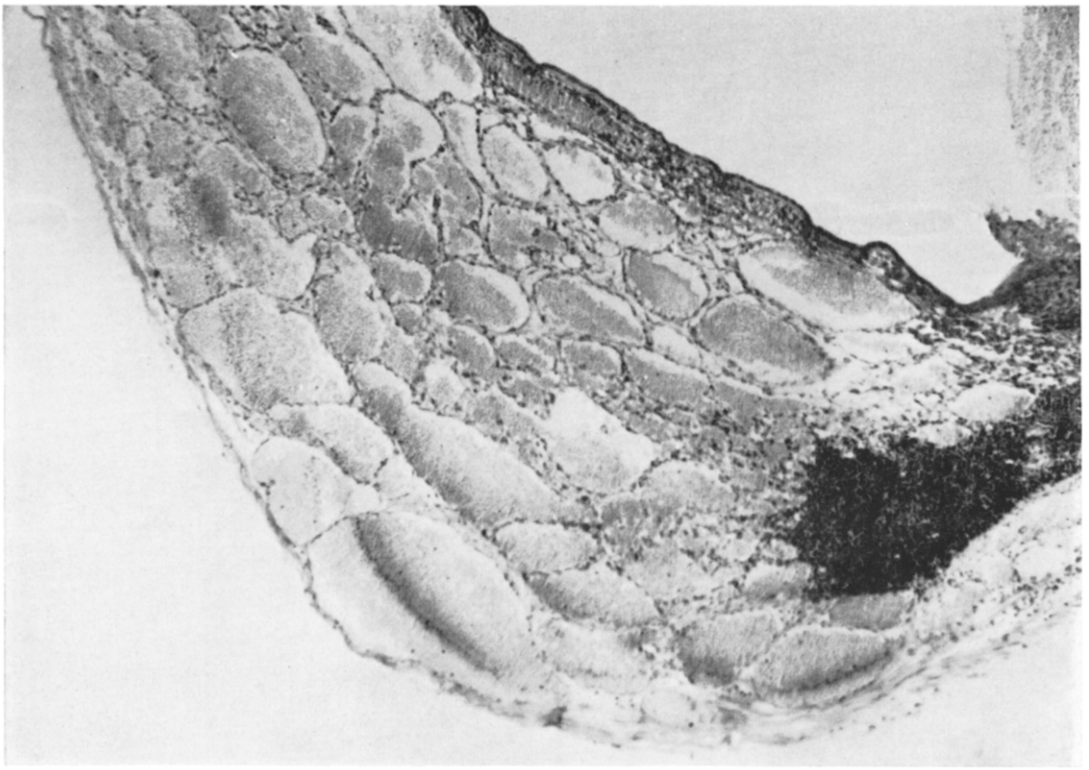

*Fig.* 3. (×25) *A mass of dilated capillaries lined by thin endothelium in the bladder wall. The bladder lumen is at the top.*

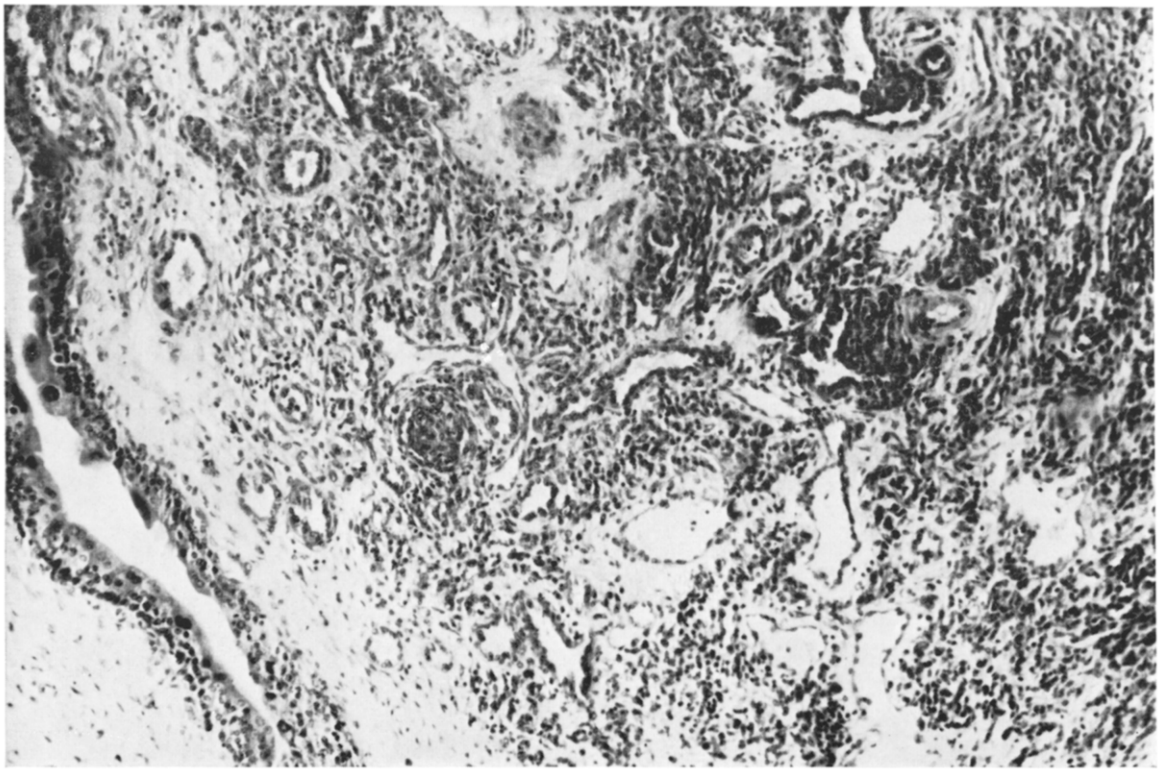

*Fig.* 4. (×63) *An area of greatly thickened bladder wall with many blood capillaries, fibroblasts and lymphocytes. The bladder lumen is on the left.*

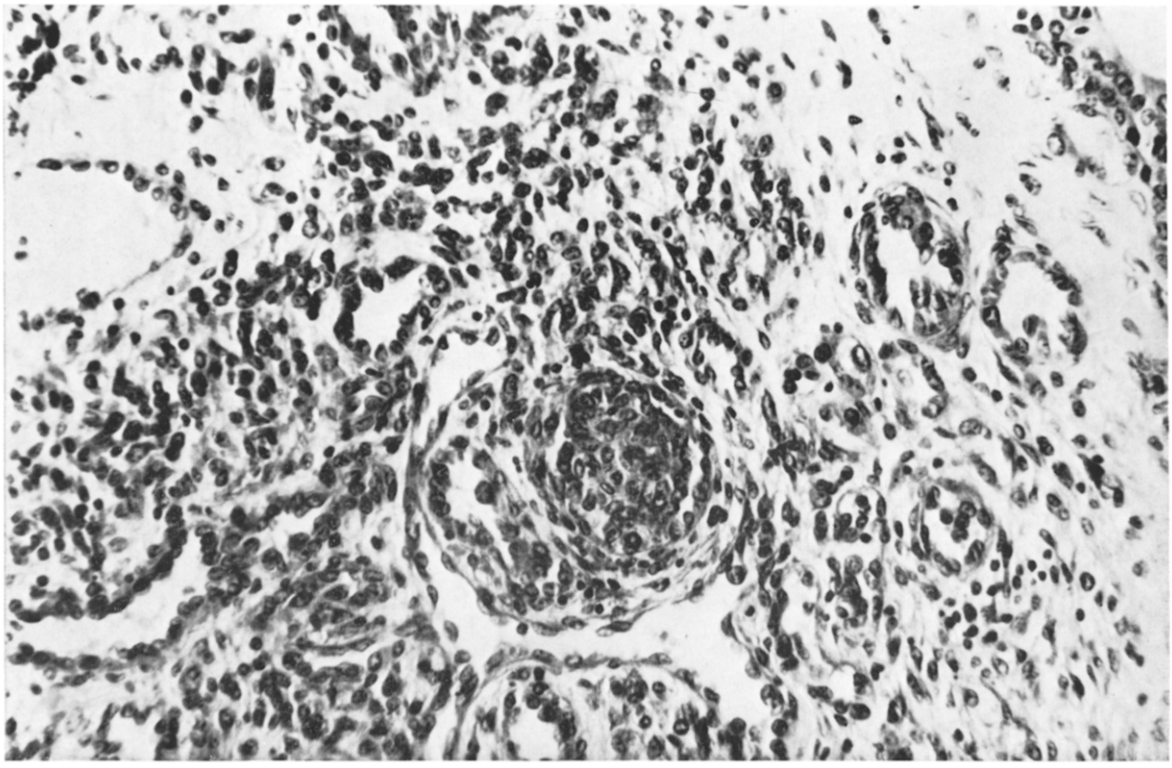

*Fig.* 5. (×157) *Area of Fig.* 4 *at a higher magnification showing a concentration of capillaries with endothelial thickening.*

tect personnel, DBNA injections were performed in an efficient fume cupboard and the mice left therein for at least 4 hr to allow unchanged DBNA to be expired. For the neonatal mice, DBNA (0·5 ml) was dissolved in 3% aqueous gelatine (25 ml) and 0·05 ml (1 µl DBNA) injected subcutaneously in the interscapular region on the first day of life and at 1 and 2 weeks thereafter. [$^3$H]-Thymidine (0·25 µCi/g] was injected intraperitoneally 1 hr before death. 2-Acetylaminofluorene (0·05%) was incorporated in the diet.

Bladder implantation was carried out in 12-week old mice by the method of Jull [20] as modified by Allen *et al.* [21]. Pellets were composed of crushed paraffin wax and weighed 15–17 mg.

Animals were killed with ether, the bladder distended with fixative and with other macroscopically abnormal tissues dissected and fixed in either Bouin's fixative or buffered formol saline. Sections were stained with haematoxylin and eosin.

DNA synthesis was measured by the method of Lawson *et al.* [15] in single bladders at intervals after the administration of DBNA.

## RESULTS

*(i) Carcinogenicity of DBNA*

Evidence for the carcinogenicity of DBNA in IF×C57 mice is presented in Table 1. Untreated IF×C57 mice develop few tumours by the age of 68 weeks. The single liver adenoma observed in 1 of 13 male mice and the absence of tumours in 17 female mice is in agreement with the previous observation of 1 hepatoma in 31 female and 0 in 19 male mice [12]. The liver tumours are classified as adenoma or hepatoma on purely morphological grounds, i.e. the degree of organisation of the tumour structure, the pleomorphism of the cells and extent of the lesion. 2-Acetylaminofluorene was administered in the diet as a positive control and by 64 weeks had induced 1 papilloma and 1 carcinoma of the bladder in 34 female mice (6%) and 4 papillomas and 7 carcinomas in 20 male mice (55%); liver tumours were present in 97% of the female and 35% of the male mice.

The subcutaneous injection of DBNA (10 µl) at two-weekly intervals was well tolerated; none of the 30 male and 30 female mice died before 28 weeks. There was slight ulceration of the injection site at the fifteenth week in a few mice and bleaching of the hair around the injection site in most mice by the 25th week. Two mice were killed after 20 weeks of treatment and were found to have hyperplastic bladder epithelium composed of small cells but without the large, often binucleate, surface cells which are characteristic of the normal epithelium. Haematuria was observed in 3 male mice at 28 weeks. On autopsy these mice had grossly thickened bladders. Further mice developed haematuria and were killed after 40, 50 and 60 weeks. As large bladder tumours were required for other investigations in the laboratory an attempt was made to keep a number of the treated animals alive for longer periods. Many animals were thus lost to the experiment as they died and were too autolysed for histological examination. There were 7 bladder carcinomas in 17 female (41%) and 1 papilloma and 6 carcinomas in 12 male mice (58%).

*Histopathology of the bladder tumours*

Many of the tumours were seen macroscopically at postmortem as white or haemorrhagic lumps projecting into and sometimes filling the bladder lumen. The tumours were sessile or pendunculated papillomas or carcinomas, basically of transitional cell type (Fig. 1) but with varying degrees of differentiation from well-differentiated non-invasive papilloma (Fig. 1) to anaplastic carcinoma (Fig. 2). There was only one squamous cell carcinoma but there were areas of squamous metaplasia with keratin formation in both tumours and hyperplastic epithelium and many mitotic figures were seen.

In contrast to the 2-AAF-treated mice, a feature of the DBNA-treated bladders was the thickening of the connective tissue due to oedema, granuloma formation and a remarkable feature was a great increase in the number of blood capillaries, often present in masses of compact or dilated and congested vessels (Fig. 3); in some bladders their concentration was so great and the endothelium so irregular and hyperplastic as to have many of the features of a haemangioma (Figs. 4, 5). An unusually large number of capillaries were seen within and concentrated around the base of the epithelial tumours.

Inflammation was more severe than in the 2-AAF-treated bladders consisting mainly of lymphocytes and plasma cells but with polymorphonuclear leucocytes and macrophages in areas of ulceration and necrosis.

*(ii) Effect of a single subcutaneous injection of DBNA (10 µl) in 12-week old IF×C57 mice*

The response of individual mice to a single subcutaneous injection of DBNA varied considerably, as has previously been described with other carcinogens [14, 15]. The earliest effect

*Table 2. The effect of a single injection of DBNA (10 µl) on the epithelium of male IF × C57 mice*

| Time after DBNA (hr) | No. of mice | Bladder epithelium | | |
|---|---|---|---|---|
| | | normal | slight hyperplasia | moderate hyperplasia |
| 24 | 4 | 4 | 0 | 0 |
| 48 | 4 | 2 | 2 | 0 |
| 72 | 2 | 1 | 1 | 0 |
| 96 | 6 | 5 | 1 | 0 |
| 120 | 4 | 2 | 1* | 1† |

*Very slight hyperplasia with increased mitotic activity.
†Increased mitotic activity.

of DBNA was to depress slightly [$^3$H-]thymidine incorporation into bladder epithelial DNA (Fig. 6). Thirty hours after the chemical had been injected there was a slow increase in [$^3$H]-thymidine incorporation to a maximum at about 72 hr, after which it declined slowly but was still elevated when the experiment was discontinued after 120 hr. At the maximum, DNA synthesis, as measured by [$^3$H]-thymidine incorporation, was about 4 times the level in the normal bladder.

Histological examination of the bladder epithelium of mice treated with a single dose of DBNA indicated that there was no demonstrable loss of epithelial cells during this time. Slight hyperplasia as judged by a patchy increase of one cell layer was apparent in some of the mice after 48 hr and moderate hyperplasia in one at 120 hr. The bladder epithelium of two mice at the latter time showed increased mitotic activity.

*(iii) Experiments in young mice*

Three injections of DBNA on days 1, 8 and 15 of life failed to induce bladder tumours in IFxC57 mice within 52 weeks (Table 3). Liver tumours were induced in 97% male and 83% female mice in this period, 4 male mice having to be killed when moribund at 51 weeks. Otherwise there was only 1 death between weaning and the termination of the experiment. The liver in male mice was more extensively affected than in female mice. There was a small number of lung tumours and a single uterine carcinoma which were not seen in control mice. Pyelonephritis and mild inflammation of the bladder were present in a few cases.

The administration of carcinogens to neonatal mice of certain strains often results in the development of "hepatomas" later in life [16, 22]. These have generally been regarded as tumours on account of their morphology and

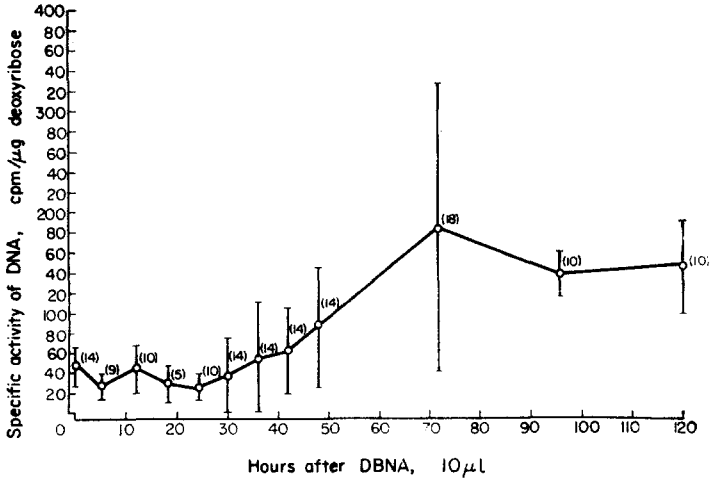


*Fig. 6. The incorporation of [$^3$H]-thymidine into DNA of the epithelial fraction of the bladder of male IF × C57 mice treated with DBNA (10 µl) the stated number of hours previously and [$^3$H]-thymidine (0·25 µCi/g) 1 hr before death. Figures in parenthesis indicate the number of mice used to determine each point. The vertical lines represent the standard deviation.*

*Table 3. The induction of tumours in IF×C57 mice following the injection of DBNA (1 μl) at 1 day, and 2 weeks of age*

| Treatment | Sex | No. of mice | Age at death (weeks) | Liver* | | | | Bladder* | | | | Lung* | | | | Other |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | adenoma | | hepatoma | | benign downgrowth | | carcinoma | | adenoma | | carcinoma | | |
| | | | | No. | (%) | No. | (%) | No. | (%) | No. | (%) | No. | (%) | No. | (%) | |
| DBNA only | F | 24 | 52 | 13 | (54) | 7 | (29) | 0 | (—) | 0 | (—) | 4 | (17) | 0 | (—) | 1† |
| | M | 37 | 52 | 4 | (11) | 32 | (86) | 0 | (—) | 0 | (—) | 12 | (32) | 3 | ( 8) | |
| None | F | 24 | 52 | 0 | (—) | 0 | (—) | 0 | (—) | 0 | (—) | 0 | (—) | 0 | (—) | |
| | M | 27 | 52 | 0 | (—) | 0 | (—) | 0 | (—) | 0 | (—) | 0 | (—) | 0 | (—) | |
| DBNA+pellet | F | 26 | 52 | 6 | (23) | 3 | (12) | 8 | (31) | 1 | ( 4) | 5 | (19) | 0 | (—) | |
| | M | 36 | 52 | 8 | (22) | 25 | (69) | 9 | (25) | 3‡ | ( 8) | 3 | ( 8) | 0 | (—) | |
| Pellet only | F | 27 | 52 | 0 | (—) | 0 | (—) | 10 | (37) | 0 | (—) | 0 | (—) | 0 | (—) | |
| | M | 32 | 52 | 0 | (—) | 0 | (—) | 5 | (16) | 2§ | ( 6) | 0 | (—) | 0 | (—) | |

*Most advanced lesion only is listed for each animal.
†Uterine carcinoma.
‡1 at urethra.
§2 at urethra.

because, after the initial treatment, they developed in the absence of further carcinogen. Levi (unpublished observations) prepared smears from 20 of the DBNA-induced liver tumours and after Feulgen staining, in which the intensity of staining is stoichiometrically related to the DNA content, measured the DNA content of 100 cells from each tumour by microdensitometery [14]. In 17 cases (16 classified as hepatoma, 1 as adenoma) the DNA distribution pattern was polyploid; the number of diploid, tetraploid, and octaploid cells being similar to that in liver from untreated mice of the same age. The other three cases (classified as hepatoma) were aneuploid; it is unlikely that the cells between the diploid, tetraploid and octaploid modes represented cells in DNA synthesis as very few mitotic figures were observed in the sections.

Fewer liver tumours were found in the chemically-treated and implanted mice (92% in male and 38% in female mice) than after treatment with the chemical alone. There were also fewer lung tumours (Table 3). The four carcinomas of the bladder in the chemically-treated and implanted mice were not significantly in excess of those induced by the implant alone (2 carcinomas). All of these carcinomas were of a low degree of malignancy (Grade 1–23) and in 3 cases arose near the urethra. Inflammation, hyperplasia and local benign downgrowth of the epithelium were common observations in the implanted mice whether or not they had been pretreated with DBNA. It is concluded, therefore, that treatment with DBNA early in life does not induce changes in the bladder epithelium of IF × C57 mice which may develop into frank carcinomas under the influence of an implanted paraffin wax pellet.

## DISCUSSION

The observations on the carcinogenic activity of DBNA, reported here, contrast with those of previous workers [18, 19]. Takayama and Imaizumi [18] found that feeding of low levels of DBNA in the diet to 1CR mice caused liver tumours, squamous carcinoma of the forestomach, lung adenomas and papillomas of the oesophagus. Bertram and Craig [19], on the other hand, using C57Bl/c mice gave the chemical in the drinking water and obtained carcinomas of the bladder and oesophagus as well as tumours of the tongue, palate and forestomach. Subcutaneous injection of DBNA into IF × C57 mice, in our hands, induced only bladder tumours. These differences probably owe more to the route of administration than to the strain of mouse. Druckrey and collaborators [1, 2] showed that in the BD rat, DBNA in the diet favoured the induction of liver and oesophageal tumours, whereas subcutaneous injection led to a higher incidence of bladder tumours. The higher yield of oesophageal, liver, stomach and oral tumours after feeding DBNA, in the diet or drinking water, suggests that these tissues may be able to activate the carcinogen locally. After subcutaneous injection, the highest effective concentration of active metabolites is probably in the urine. It is difficult to understand why this should occur with DBNA and not with other carcinogenic nitrosamines.

The haemangiomatous lesions found in the bladder of mice injected subcutaneously with DBNA were not induced when this chemical was given in the drinking water [19]. Haemangiomas have, however, been described in the bladder of cattle exposed to bracken fern (*Pteris aquilina*) in the diet [24]. It is not clear whether the DBNA-induced lesions arose as a result of non-specific reaction to tissue injury or are a direct consequence of the action of the chemical.

The induction of hepatomas in mice treated with DBNA when young emphasizes the importance of the experimental conditions on the site of tumours induced by this chemical. Neonatal mice differ from adults in their immunological competence, the absence of certain metabolic enzymes and in the degree of cellular proliferation in various tissues [16]. It is not certain, on present evidence, which of these factors is of importance in defining the distribution of tumours with DBNA. The failure of mice treated with this chemical in early life to develop bladder tumours even when a paraffin wax pellet was implanted into the lumen to provide a proliferative stimulus suggests that the chemical, under these conditions, has little effect on the bladder epithelium and, in consequence, that its metabolic activation is affected. Previous attempts to render the bladder epithelium of adult mice susceptible to the induction of tumours by the implantation of a paraffin wax pellet were successful when a limited amount of 2-aminodiphenylene oxide was employed [12] although recent experiments using cyclophosphamide, *ortho*-aminoazotoluene and xanthurenic acid-8-methyl ether were not [25].

Although DBNA, ENS, 2-acetylaminofluorene and 2-aminodiphenylene oxide all induce urinary tract hyperplasia on oral administration to mice for 2–12 weeks [11, 13] there is an important difference in their acute effect on the

bladder. The acute effect of ENS is characterized by the early onset of an extensive proliferative reaction accompanied by cellular necrosis and desquamation of the bladder epithelium whereas neither DBNA nor AAF induces as rapid or as intense a response [15]. It is therefore apparent that the induction of a massive intitial cellular proliferation is not essential for the induction of bladder tumours and may be contrary to the general pattern of bladder carcinogenesis in animals.

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## SUMMARY

*Subcutaneous injection of dibutylnitrosamine (DBNA) into adult male and female IF×C57 mice led to the induction of carcinomas of the epithelium of the urinary bladder. Haemangiomatous lesions were also present in these bladders. A single injection of DBNA led to an increase in $^3$H-thymidine incorporation into bladder epithelial DNA starting at 30 hr after injection and attaining a maximum at 72 hr. Slight hyperplasia was observed in some bladders 48 hr after injection and increased mitotic activity after 120 hr. Liver tumours but no bladder tumours were induced when DBNA was injected on days 1, 8 and 15 of life and the mice allowed to survive to 52 weeks without further treatment. The implantation of a paraffin wax pellet into the lumen of the bladder of mice treated at an early age with DBNA did not lead to a significant incidence of bladder tumours. These results are considered from the view-points of the tumour localization and their relationship to bladder carcinogenesis.*

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# Experimental Chemotherapy of a Transplantable Rhabdomyosarcoma in the Rat*

G. FRANCHI, L. MORETTI and S. GARATTINI

*Istituto di Ricerche Farmacologiche "Mario Negri", 62 Via Eritrea, 20157 Milan, Italy and European Organization for Research on Treatment of Cancer (EORTC)*

## INTRODUCTION

SEVERAL investigations have demonstrated that a rhabdomyosarcoma which arose in 1962 in the mandibula of a rat radiation chimera is a radio-resistant tumor [1, 2]. It was therefore interesting to study the sensitivity of this neoplastic disease to a number of antitumoral drugs. The experiments hereafter described were performed with the Rhabdomyosarcoma BA 1112 previously mentioned which is isologous in WAG/Rij inbred strain of rats.

## METHODS

The tumor and the 12–15 weeks old female rats were kindly supplied by Dr. D. van Bekkum, Radiobiological Institute, TNO, Rijswijk, The Netherlands. The tumor was excised in aseptic conditions, cut in fragments of about 50 mg and transplanted subcutaneously by trocar in the right intrascapular area of rats. Thirty days after transplantation two diameters of the tumors were measured and the tumor volumes determined according to Gelzer [3]. The mean tumor volume at the beginning of the treatment was $2{\cdot}6 \pm 0{\cdot}3$ cm$^3$.

## RESULTS

Three groups of compounds have been chosen to be tested on this experimental tumor: alkylating agents, antimetabolites, 6-aminochrysene and its derivatives. For the compounds of the first two groups the doses employed represent 1/6 of the $LD_{50}$ determined after 6 consecutive days of treatment by intraperitoneal administration on Sprague-Dawley rats, for the derivatives of 6-aminochrysene the doses were arbitrarily chosen. The treatments were carried out by intraperitoneal or oral administration on the following days: 1, 2, 3, 4, 5, 6, 7, –, 9, –, –, 12, –, 14, –, 16, beginning 30 days after tumor transplantation. On the 19th day the animals were sacrified and the weight of their tumor was measured. The body weight and spleen weight were also recorded as an index of toxicity. Table 1 reports the effect of well known antitumoral agents on the growth of Rhabdomyosarcoma BA 1112. It is remarkable that several alkylating agents as well as antimetabolites, tested at nearly toxic doses, do not show any inhibitory effect on the tumoral growth. On the contrary, results reported in Table 2 show a therapeutic effect of a series of derivatives of 6-aminochrysene.

## CONCLUSION

These experimental observations confirm the carcinostatic properties of 6-aminochrysene and its derivatives evaluated on methylcholanthrene induced tumors [4], on spontaneous mouse carcinoma [5, 6] and human mammary cancer [7]. Since 6-aminochrysene derivatives are inactive on transplantable mouse tumors (Sarcoma 180 and Ehrlich carcinoma) [6] the need to use new experimental tumors for detecting new anticancer agents is apparent.

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*Table* 1. *Effect of different antitumoral drugs on Rhabdomyosarcoma BA* 1112 *transplanted subcutaneously in Wag/Rij rats*

| Compounds | Dose (mg/kg/ i.p.) | Av. body weight (g±S.E.) | Av. tumor (g±S.E.) | Tumor weight inhibition % | Spleen weight (g±S.E. 100 g body weight) % |
|---|---|---|---|---|---|
| Saline | — | 210±0·5 | 40·2±2·4 | — | 0·326±0·01 |
| Cyclophosphamide | 8 | 205±0·7 | 34·4±5·1 | 15 | 0·408±0·08 |
| DL Sarcolysine | 2·1 | 203±0·4 | 37·0±3·2 | 9 | 0·281±0·01 |
| Triethylenemelamine (TEM) | 0·12 | 198±0·2* | 33·0±4·7 | 18 | 0·417±0·05 |
| Mannitol mustard | 5 | 209±0·4 | 39·9±4·2 | 1 | 0·345±0·02 |
| Methotrexate | 0·17 | 215±0·2 | 43·9±4·4 | 0 | 0·388†±0·02 |
| 5-Fluorouracil | 10 | 210±0·2 | 43·1±5·5 | 0 | 0·451†±0·04 |
| 6-Mercaptopurine | 25 | 206±0·1‡ | 37·1±2·8 | 8 | 0·649*±0·05 |

Each group consisted of 10 rats. All compounds were dissolved in saline.
*P<0·01.
†P<0·05.
‡20% mortality.

*Table* 2. *Effect of* 6-*aminochrysene and its derivatives on Rhabdomyosarcoma BA* 1112 *transplanted subcutaneously in Wag/Rij rats*

| EORTC No. | Dose (mg/kg/ i.p.) | Av. body weight (g±S.E.) | Av. tumor weight (g±S.E.) | Tumor weight inhibition % | Spleen weight (g±S.E.) % |
|---|---|---|---|---|---|
| Saline | — | 212±0·4 | 25·1±4·1 | — | 0·379±0·2 |
| 116 | 100 | 165±11* | 6·3±0·5§ | 75† | 0·413±0·3 |
| | 50 | 187±0·5* | 11·0±2·4§ | 55† | 0·205±0·2 |
| | 100‡ | 193±0·4 | 13·6±1·3 | 45† | 0·340±0·3 |
| 118 | 100 | 179±12 | 10·3±0·1¶ | 59 | 0·505±0·4 |
| 334 | 100 | 189±0·5* | 8·8±0·5 | 65† | 0·389±0·3 |
| | 50 | 190±0·3* | 10·5±1·3§ | 58† | 0·293±0·1 |
| | 100‡ | 200±0·3 | 18·9±3·6§ | 24 | 0·335±0·1 |
| 357 | 100 | 204±0·4 | 27·9±2·9§ | — | n.d. |
| 500 | 100 | 200±7 | 12·8±2·2 | 49* | 0·422±0·3 |
| | 50 | 211±0·6 | 14·5±2·6§ | 42† | 0·390±0·3 |
| | 100‡ | 199±0·2 | 12·4±1·4 | 50† | 0·377±0·1 |
| 502 | 100 | 210±0·1 | 12·1±0·8 | 52† | n.d. |
| | 50 | 204±0·6 | 18·7±4 | 25 | 0·394±0·3 |
| | 100‡ | 197±0·3 | 15·5±1 | 38† | 0·313±0·1 |
| 504 | 100 | 204±0·7 | 18·5±1·9§ | 26 | n.d. |
| 578 | 100 | 200±1·2 | 9·9±0·9 | 61† | n.d. |
| | 50 | 198±0·2 | 12·7±1·7 | 49† | 0·358±0·2 |
| | 100‡ | 204±0·5 | 15·0±3·4 | 40† | 0·358±0·2 |
| 579 | 100 | 226±2 | 18·2±1 § | 27* | n.d. |
| 581 | 100 | 218±0·8 | 12·1±1 | 52† | n.d. |
| | 50 | 197±0·7 | 19·1±2·9 | 23 | 0·379±0·2 |
| | 100§ | 197±0·5 | 15·0±2·4 | 40† | 0·362±0·1 |

Each group consisted of 10 rats. All compounds were dissolved in peanut oil.
n.d.=not determined.
*$P<0·05$.
†$P<0·01$.
‡Given orally.
§10% mortality.
¶40% mortality.

Chemical formula of EORTC compounds:

116—6-aminochrysene: 118—N-(6-chrysenyl)-carbamic acid ethyl ester; 334—6-chrysenaminomethane sulphinic acid, Na salt; 357—N-(N-chrysenyl)-carbamic acid, hexadecyl ester; 500—N piperidinoacetyl-6-chrysenamine; 502—N-butilaminoacetyl-6-chrysenamine; 504—N-diethyl aminoacetyl-6-chrysenamine; 578—N-(4-hydroxy-3-methoxy benzylidene)-6-chrysenamine; 579—N-(6-chrysenyl) oxamic acid, ethyl ester; 581—N-(4-di-methyl-aminobenzylidene)-6-chrysenamine.

## SUMMARY

*The growth of rhabdomyosarcoma BA* 112 *transplanted in WAG/Rij inbred rats is reduced by the administration of* 6*-aminochrysene and several derivatives.*

*The activity is remarkable considering that several alkylating agents and antimetabolites are without effect on the growth of this tumor.*

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# Phosphorylcholine: A Tumour-Inhibitory Substance Extracted from Seminal Plasma

RACHEL STEIN-WERBLOWSKY
*Royal Veterinary College, London, N.W.1, England*

## INTRODUCTION

THE PRESENCE of a tumour-inhibitory factor in the post-coital uterine fluid of the rat has already been demonstrated [1]. The present paper extends earlier observations and describes experiments designed to isolate the active constituent from such fluid, to determine its chemical composition and to investigate its mode of action. Evidence will be presented to show that the tumour-inhibitory factor is a phospholipid which enhances the immunological competence of the tumour cell.

## MATERIAL AND METHODS

*Rats*

White Wistar rats, weanlings of 80–100 g, adults of 150–180 g, pregnant females and adult cavies of 500 g were obtained from Messrs. Tuck & Son, Rayleigh, Essex.

*Biological material*

The amounts of post-coital fluid which could be collected from laboratory animals other than rats, such as mice, hamsters, guinea pigs and rabbits, were found to be insufficient for this investigation. Specimens of sow post-coital fluid were obtained from the Animal Research Laboratories of the Wellcome Foundation, Frant, who also kindly supplied specimens of boar, ram, bull, jackass, stallion, mule, dog and cock semen.

*Preparation of extracts from biological fluids*

Post-coital fluid and semen specimens were mixed with 10 volumes methanol/chloroform 1:1 and brought to the boil. The precipitate formed was filtered off and the liquid extract evaporated to dryness. The residue in the flask was suspended in various volumes of saline. All except the bull-semen extracts were found to have pH values from 7·4–7·6. The pH of bull semen was adjusted with $NaHCO_3$.

*Chemicals*

Choline, phosphorycholine (PC) and glycerylphosphorylcholine (GPC) were obtained from Messrs. Light & Co., Colnbrook, Bucks. They were prepared as solutions of 1, 2, 3, 5 7 and 10% in normal saline. The pH values of these substances were within physiological limits except that of GPC, which was adjusted with $NaHCO_3$. Trypsin was supplied by British Drug Houses, Poole, and made up as a 0·2% solution in saline.

*Tumours*

A transplanted tumour, originally chemically induced in the rat uterus in 1960 [2]-was maintained by passage in white Wistar rats. The tumour grew at all sites and killed the host in 2–3 weeks. The Walker rat tumour was obtained from the Chester Beatty Research Institute, London, and the Dael guinea-pig tumour from The Imperial Cancer Research Fund, London.

*Preparation of tumour suspensions*

One volume of tumour was minced in two volumes of either saline or trypsin/saline in a

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glass grinder (15 cm long, clearance 1·0 mm, manufactured by Messrs. Aimer, London). Incubation with trypsin was carried out at room temperature (20–22°C) with occasional stirring, for 30 min. The suspension was decanted into centrifuge tubes, leaving the large fragments behind, centrifuged at 1500 rev/min for 10 min and resuspended in saline or in the substance under test.

*Methods of injection*

An amount of 0·2–0·4 ml of tumour suspension was injected subcutaneously or intradermally into the flank of weanling rats, and doses of 0·3–0·4 ml subcutaneously or intradermally into the abdominal skin of adult rats. The guinea-pigs received 0·2 ml of Dael-tumour suspension into the flank. For intracerebral inoculation no more than 0·05 ml of tumour suspension was injected. Neonates received 0·05–0·1 ml into the nuchal region.

For intra-uterine inoculation, the tumour suspension was centrifuged and the cells concentrated in the button aspirated with a wide bore needle and inoculated into the uterine horn without pressure. A ligature was placed above the injection site to ensure retention of the inoculum. A second injection was made intradermally into the same recipient.

All the tumours that took, killed the host within 2–4 weeks. They are listed as "tumour positive". Animals that were free of tumour at the end of the experiment are classified as "tumour negative". Animals which died from causes other than tumour have been excluded from the Tables.

*Test for anti-tumour activity*

Tumour suspensions were incubated with substances under test, i.e. semen, semen extracts, choline, PC and GPC for 1–5 hr at room temperature. At the end of this period, the suspension was centrifuged again, the supernatant discarded and the sediment resuspended in saline to make up the original volume. Control tumours were treated in a similar manner except that saline was used instead of the test substance. The quantity of the biological fluid was the minimum consistent with tumour inhibition. Lower values are not included in the Tables. After incubation, tumour cell suspensions were injected subcutaneously into weanling rats.

Some experiments were repeated with tumour cells heat killed by exposure to 56°C for 30 min.

*Determination of cell viability*

Viability of tumour cells *in vitro* was assessed by phase microscopy and by histology.

Cells exposed to PC and controls were fixed in Bouins and stained with haematoxilyn-eosin. Injection sites of experimental and control animals were excised at various intervals after tumour inoculation and treated as above.

## RESULTS

*Chemical analysis*

Tumour inhibitory activity of the rat post-coital fluid was not destroyed by heating to 56°C, freezing to −79°C, or storage up to 3 weeks at −20°C. Desiccation and lyophilization reduced activity. The tumour-inhibitory factor was not water soluble, but could be extracted with methanol/chloroform. This extract, when dried, was water soluble and withstood heating to 100°C. Paper chromatography of the methanol/chloroform extract revealed the presence of substances between choline and acetycholine.

*Determination of the active fraction*

Post-coital fluid is a composite substance consisting of spermatozoa, seminal plasma, oestrus fluid and possible conjugates of these constituents. Previous experiments [1] had shown that neither washed spermatozoa, nor oestrus fluid, nor post-coital fluid from rats mated with vasectomized males, contained the inhibitory factor. The active constituent, therefore, seems to derive from that fraction of the seminal plasma which is produced by the epididymis.

In continuation of these experiments sow post-coital fluid and semen specimens from various species were extracted with methanol/chloroform and tested for tumour-inhibitory activity. The findings, listed in Table 1, show that extracts of seminal plasma of various species contain the inhibitory factor in various concentrations. It is of interest to note that fresh semen or seminal plasma was inactive.

The data on the inhibitory factor may thus be summed up:

(1) It is present in the seminal plasma and possibly secreted by the epididymis;
(2) Activity is only "unmasked" when seminal plasma is exposed to the uterine environment or extracted with methanol/chloroform.
(3) Paper chromatography of the methanol/chloroform extract indicates a choline containing substance.
(4) The inhibitory activity is in direct rela-

*Table 1. Tumour growth in weanling rats. Tumour inocula exposed to semen or seminal extracts prior to injection*

| Specimen | Induced tumour | | | | | Walker tumour | | |
|---|---|---|---|---|---|---|---|---|
| | GPC mg/100 ml semen. Table Mann | Proportion v/v specimen/unit volume tumour | Experimental tumour bearing animals | Inhibition % | Control tumour bearing animals | Experimental tumour bearing animals | Inhibition % | Control tumour bearing animals |
| Rat post-coital fluid extracted | 2380 | 3–10 | 5 (64)* | 78 | 39 (40)* | | | |
| Fresh ram semen — unextracted | 1185 | 3 | 5 (5)* | 0 | 13 (15)* | | | |
| Ram semen — extracted | | 3–5 | 1 (14)* | 71 | | 11 (53)* | 76 | 28 (34)* |
| Bull semen — extracted | 496 | 20–40 | 3 (9)* | 66 | 10 (10)* | | | |
| Rabbit semen — extracted | 215–270 | 10–20 | 4 (13)* | 69 | 3 (3)* | 4 (4)* | 0 | 2 (2)* |
| Jackass semen — extracted | — | 20–40 | 0 (6)* | 100 | 7 (7)* | | | |
| Dog semen — extracted | 180 | 20–40<br>50 | 1 (33)* | 97 | 10 (13)* | 2 (2)* | 0 | 2 (2)* |
| Boar semen — extracted | 108–235 | 40<br>80 | 7 (44)* | 85 | 58 (61)* | 7 (10)*<br>5 (12)* | 30<br>58 | 1 (10)* |
| Stallion semen — extracted | 38–113 | 40 | 2 (12)* | 84 | 17 (18)* | 4 (13)* | 70 | 6 (6)* |
| Mule semen — extracted | — | 40 | 0 (3)* | 100 | 2 (3)* | 0 (5)* | 100 | 2 (2)* |
| Domestic cock semen — extracted | PC+ | 20 | 0 (2)* | 100 | 2 (2)* | | | |
| Man semen — extracted | 59 PC+ | 15 | 0 (2)* | 100 | 2 (2)* | | | |

*Total number of animals

tion to the GPC or PC content of the seminal plasma as tabulated by Mann [3].

The inhibitory factor thus appears to be a phospholipid secreted by the epididymis, i.e. a breakdown product of a GPC or PC complex.

To test this hypothesis, three phospholipids, choline, PC and GPC were examined for their ability to inhibit the growth of the induced tumour as well as that of the Walker and Dael tumour. The high price of GPC has precluded its more extensive use at this stage.

Table 2 shows that the induced tumour was readily inactivated by GPC and PC, whereas the Walker tumour only showed a good response when pre-treated with trypsin. The non-trypsinized Dael tumour was not inhibited, even at a concentration of 10% PC.

*Mode of action*

GPC and PC are normal constituents of the epididymis and of the seminal plasma and are not known to have any adverse effect on the uterus, but tumour cells, when incubated with these substances, fail to grow on transplantation. These phospholipids, therefore, either kill the tumour cells or induce cellular alterations which result in their loss of transplantability.

*Determination of cell viability*

Microscopic examination of tumour cells, exposed to PC, showed that these cells form large aggregates which can only be broken up with difficulty. Dissociated cell tended to reaggregate and cell counts were unreliable. However, it would appear that PC as such is not a cytotoxic substance. Fifty-seven per cent of the non-trypsinized Walker tumours and 89% of the non-trypsinized Dael tumours took, even though the latter was incubated with a 10% solution of PC for 5 hr.

Histological examination of cells, exposed to PC, showed no alteration in cellular morphology. The subcutaneous sites, excised 3, 8 and

*Table* 2. *Action of phospholipid on tumour growth. Tumour exposed to phospholipid prior to inoculation into weanling rats*

| Tumour | Trypsin treated | Phospholipid | Concentration of PL in saline | Time of incubation (hr) | *Experimental* tumour bearing animals | *Control* tumour bearing animals |
|---|---|---|---|---|---|---|
| Induced | — | Choline | 1+2% | 5 | 9 (10)* | 12 (15)* |
| | — | Choline | 3% | 5 | 10 (10)* | |
| | — | Choline | 5% | 5 | 2 (4)* | 2 (4)* |
| Induced | — | GPC (free acid) | 1% | 5 | 0 (16)* | 29 (36)* |
| | | GPC (Na salt) | 1% | 5 | 4 (31)* | |
| Induced | — | PC | 2% | 1 | 8 (8)* | 16 (16)* |
| | — | PC | 5% | 1 | 18 (28)* | |
| | — | PC | 2% | 5 | 19 (154)* | 55 (69)* |
| | — | PC | 5% | 5 | 14 (204)* | 162 (180)* |
| Induced | + | PC | 2% | 1 | 30 (33)* | 29 (30)* |
| | + | PC | 5% | 1 | 5 (61)* | |
| | + | PC | 2% | 5 | 0 (7)* | 7 (7)* |
| Walker | — | GPC | 1% | 5 | 9 (9)* | 9 (9)* |
| Walker | — | PC | 2% | 1 | 4 (4)* | 13 (16)* |
| | — | PC | 5% | 1 | 13 (17)* | |
| | — | PC | 1–2% | 5 | 25 (37)* | 23 (36)* |
| | — | PC | 5% | 5 | 29 (51)* | 56 (58)* |
| Walker | + | PC | 2% | 1 | 2 (4)* | 13 (16)* |
| | + | PC | 5% | 1 | 6 (29)* | |
| | + | PC | 2% | 5 | 0 (4)* | 4 (4)* |
| Dael | — | PC | 4–10% | 5 | 16 (18)* | 14 (17)* |

* = Total number of animals
PL = Phospholipid

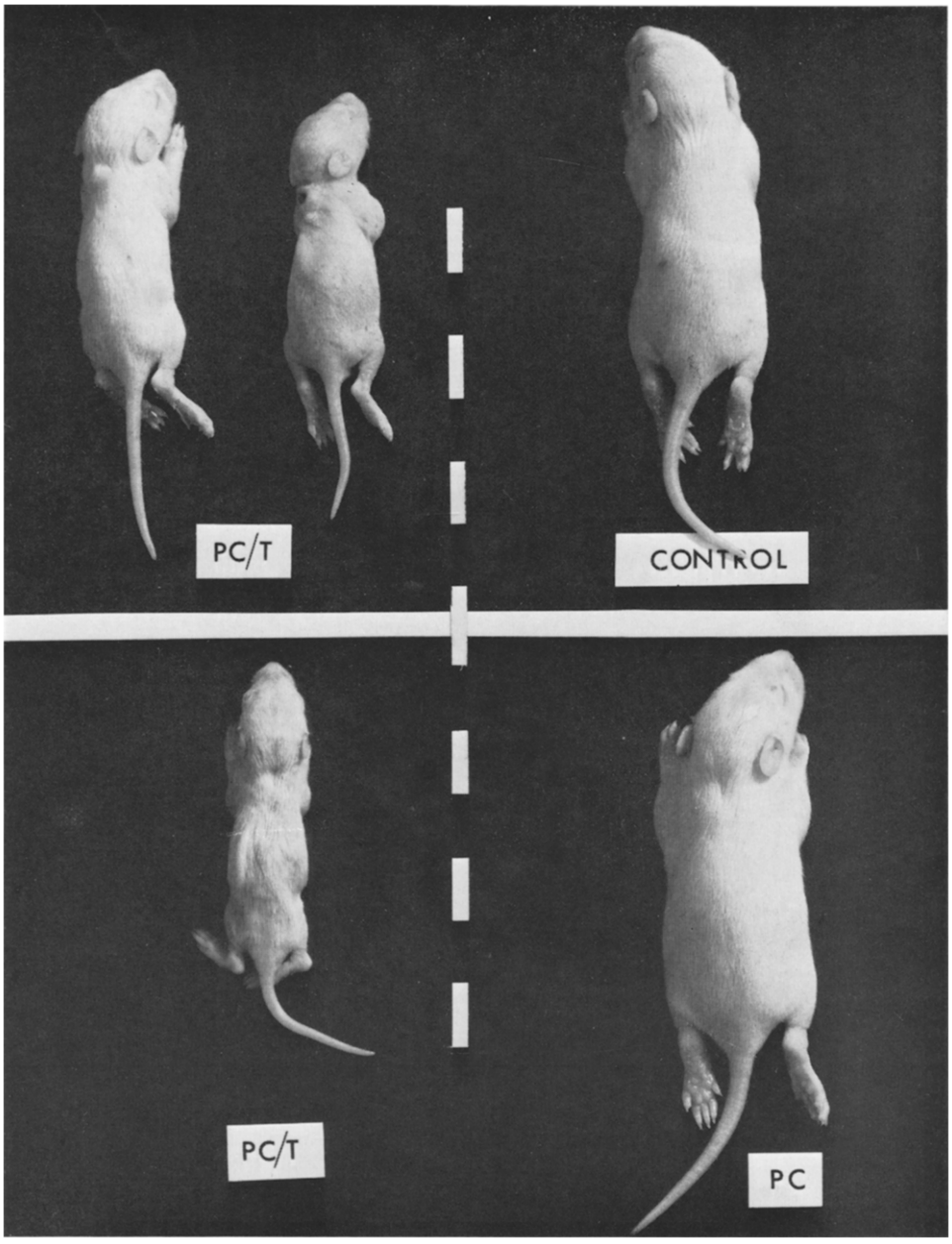


*Fig.* 1. *Littermates injected at* 1 *day of age. Top row. Left: injected with PC/tumour conjugate. Runting. Right: Control. Normal size. Bottom row. Left: Injected with PC/tumour conjugate. Severe runting. Right: Injected with PC solution. Rat larger than control.*

(to face p. 448)

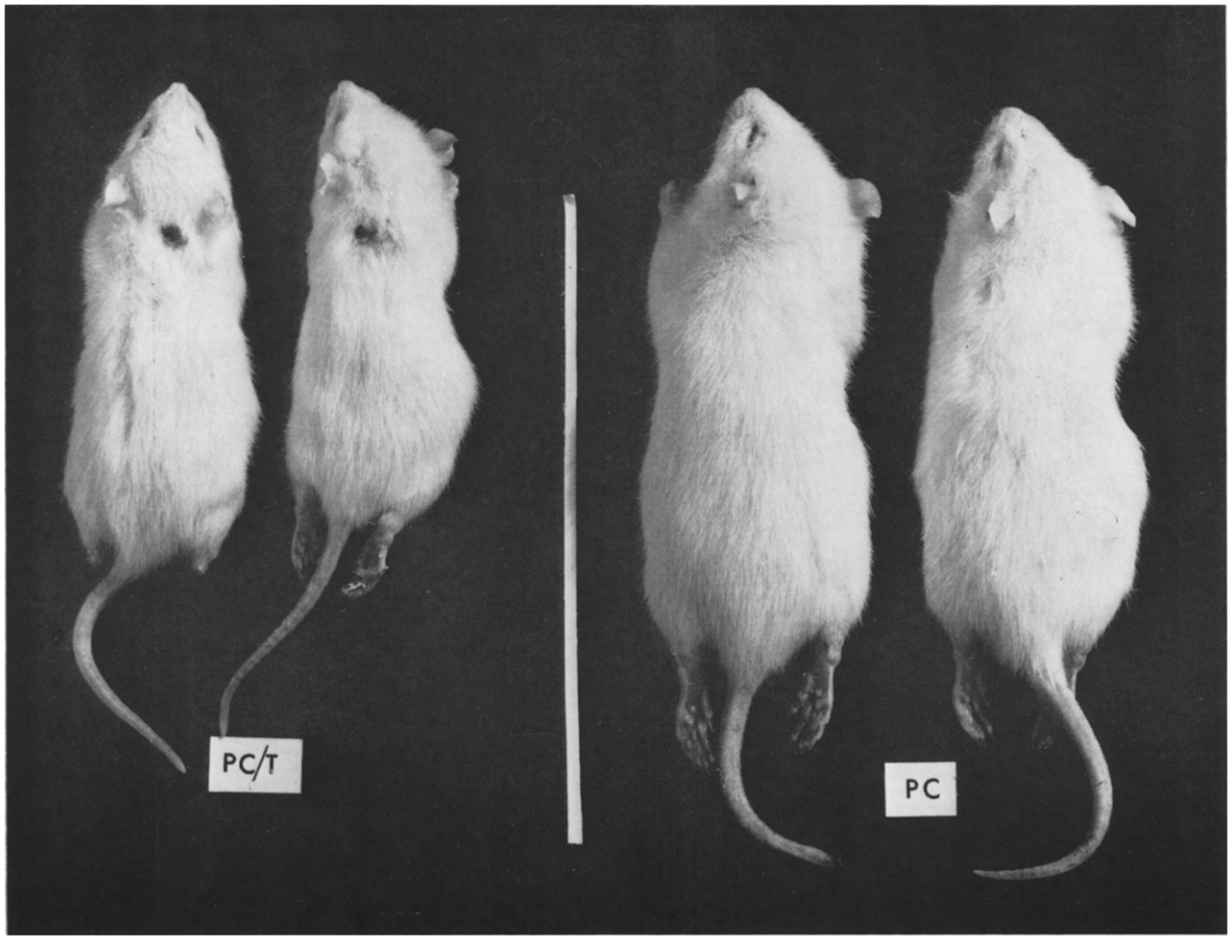


*Fig. 2. Littermates 3 weeks old, injected at 1 day of age. Left: Injected with PC/tumour conjugate. Ulcer at injection site. Runting. Right: Controls—normal size.*

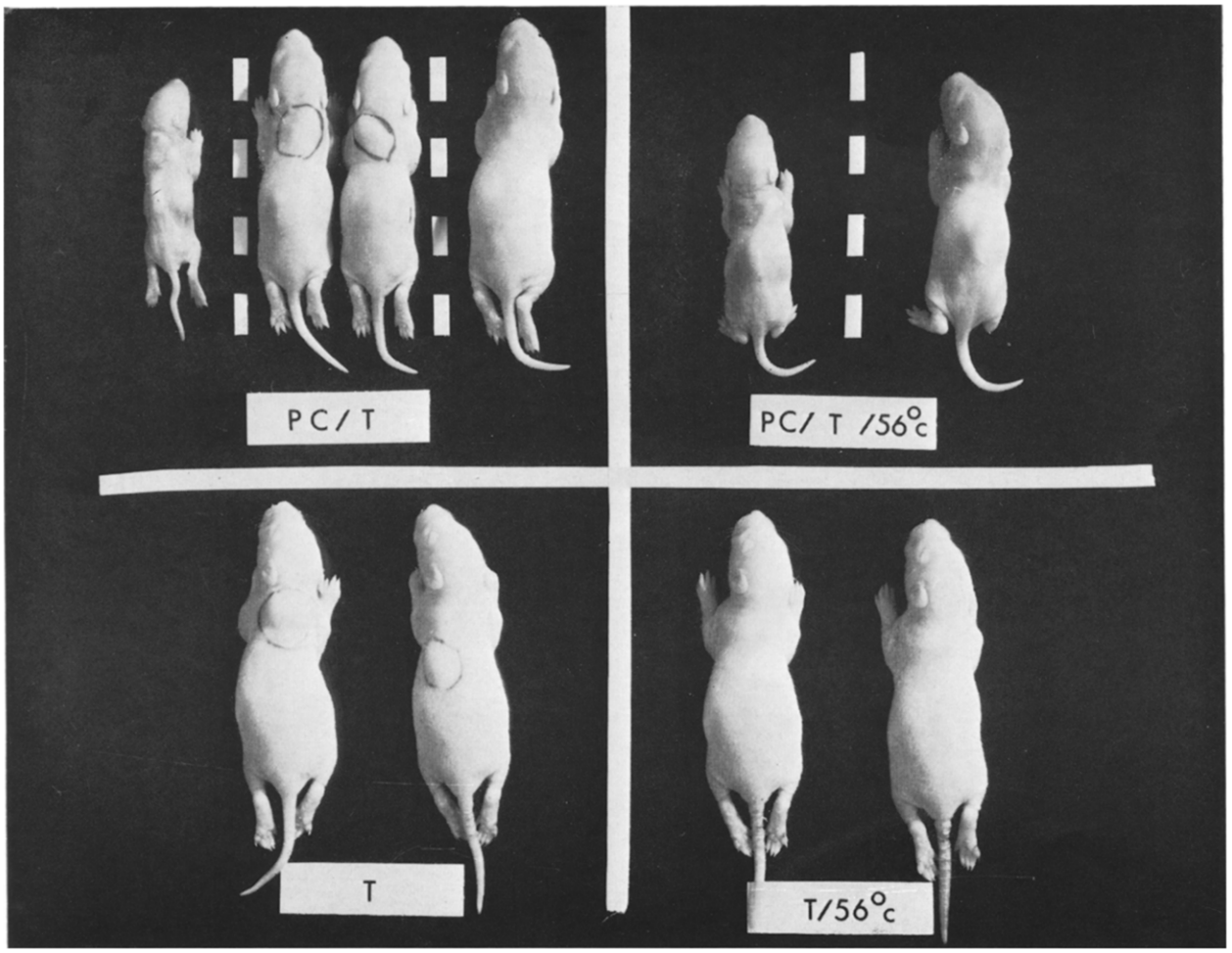


*Fig.* 3. *Littermates injected at* 1 *day of age. Top row. Left: Injected with PC/tumour conjugates — 1st rat: Severe runting. 2nd and 3rd rat: Tumour growth, no runting. 4th rat: No tumour, no runting. Right: Same suspension heat inactivated: Runting in both animals. Bottom row. Left: Injected with live tumour: Tumour growth, no runting. Right: Injected with heat killed tumour: No tumour growth, no runting.*

*Table* 3. *Injection of induced tumour/PC conjugates into recipients of various ages*

| Age | Site of injection | Inoculum and amounts | *Experimental* runting | (PC/tumour conjugates) Tumour+ | Tumour− | *Control* runting | (Tumour only) Tumour+ | Tumour − |
|---|---|---|---|---|---|---|---|---|
| 0–2 days | Subcutaneous nuchal region | 0·05–0·1 ml | 155 (42%) | 115 (35%) | 59 (23%) | — | 48 (81%) | 11 (19%) |
| | | 0·05–0·1 ml heat killed | 7 (33%) | 0 | 14 (66%) | — | 0 | 11 (100% |
| 3–5 days | Subcutaneous nuchal region | 0·05–0·1 ml | 28 (17%) | 80 (55%) | 54 (28%) | | | |
| Weanling | Subcutaneous flank | 0·3 ml | — | 9 (13%) | 59 (87%) | — | 34 (84%) | 7 (16%) |
| Adult | Brain | 0·05 | — | 49 (69%) | 22 (31%) | — | 51 (89%) | 6 (11%) |
| (same) | id | 0·4 | — | 0 | 23 (100%) | — | 12 (100%) | — |
| Adult | Intrauterine | Concentrated | — | 4 (36%) | 7 (64%) | — | 8 (100%) | 0 |
| (same) | id | | — | — | 5 (100%) | — | 8 (100%) | 0 |
| Adult | id | 0·4 ml | — | 0 | 27 (100%) | — | 15 (100%) | 0 |

Tumour+ = Tumour present
Tumour− = Tumour absent
id = intradermal

24 hr after tumour inoculation, showed increasing degrees of white cell infiltration and necrosis. In the sections from control animals, there was tumour growth and a moderate white cell reaction.

*Determination of cell-transplantability*

Tumour/PC conjugates were injected into various sites of rats of various ages. Table 3 indicates that the induced tumour, exposed to PC, failed to grow in 100% of the adults and in 87% of the weanlings when inoculated subcutaneously or intradermally. Intracerebral inoculation gave rise to tumours in 69% of the recipients, but intradermal injection did not result in tumour growth, even though the dose was increased eightfold. Intra-uterine inoculation of concentrated tumour yielded growth in 36% of the test animals, in none of which the intradermal tumour grew.

In 1–2 day old neonates the PC/tumour conjugates grew in 35% of the animals while 42% showed runting (Figs. 1–3). Runting occurred to a lesser degree, when PC/tumour suspensions were injected into neonates 3–5 day old. Correspondingly, there was an increase in tumour incidence. In animals older than 5 days, the PC/tumour conjugate was rejected and runting was not observed.

Injection of PC solution (0·1 ml of a 1–2% solution in saline) or of heat killed tumour caused no runting in neonates, but administration of heat-inactivated PC/tumour conjugates brought about wasting in new-born rats. (Fig. 3).

## DISCUSSION

The foregoing experiments establish that the tumour-inhibitory factor, originally found in the rat post-coital fluid, is derived from the epididymal portion of the seminal plasma. It is a choline containing phospholipid, probably PC or GPC. The epididymis has been shown to be an important source of GPC [4]. This substance is produced in a conjugated form and can only be activated by treatment with methanol/chloroform or by exposure to the uterine environment. Uterine fluid, particularly during the oestrus phase, contains an enzyme which hydrolizes seminal GPC [5], a fact which explains tumour inhibitory activity in the post-coital fluid and its absence from fresh semen.

As to the mode of action of PC on tumour, the findings in Table 3 demonstrate that PC is not altogether lethal to tumour cells, since PC/tumour conjugates could grow in the adult brain, in neonates and in the uterine horn. Yet, intradermal inoculation into the same adult recipients elicited a white cell infiltration with necrosis of the inoculum.

The brain is known to be an immunologically privileged site accepting homografts [6]. The PC/tumour conjugate thus behaves like a non-malignant homograft which grows in the brain but not in intradermal or sub-cutaneous tissue.

The PC/tumour conjugate also gave rise to tumours in neonates which are immunologically immature. Table 3 demonstrates that the graft–host relationship undergoes variations *pari passu* with the immunological maturation

of the host. PC/tumour conjugates, inoculated within 48 hr of birth cause runt disease or tumours in a large proportion of neonates (Fig. 3). Runting and tumour growth exclude each other. Between the 2nd and 5th day, a decrease in runting and an increase in tumour incidence becomes evident. In animals older than 5 days, immunocompetence is fully developed and the host is able to reject the PC/tumour conjugate.

The uterus too, may be regarded as an immunologically privileged site which allows the implantation of the conceptus on the 5th day after mating. The intra-uterine tumour growth in virgin rats is to be attributed to the presence of GPC diesterase in the uterine fluid [5]. This enzyme, if present in sufficient amounts, removes the PC from the PC/tumour conjugate, leaving the tumour free to grow as in the controls.

The rejection of PC/tumour conjugates in sub-cutaneous or intradermal sites of adult rats may well be ascribed to an immunological mechanism. PC would then induce immunity against tumour and act as "Freund's adjuvant" [7–9]. Alternatively, PC may be incorporated in the membrane of the tumour cell and convert it into an immunologically competent unit. The phenomenon of runting (Table 3) supports the latter interpretation since runt disease in neonates indicates the presence of immunologically competent cells in the inoculum [10].

Neither live or heat-killed tumour cells nor PC solution alone, induced any runting in neonates whilst PC/tumour conjugates, heat inactivated or live, carried a high morbidity and mortality due to wasting (Fig. 3). Membrane-associated antigens, in particular transplantation antigens, can be detected in live or waterlysed cells [11]. These antigens would cause runting in neonates.

One of the characteristics of tumour cells is their apparent loss of tissue-specific antigens from the cell surface [12–15]. Antigens of this kind are phospholipoproteins. On the other hand, tumour cells are deficient in choline containing phospholipids [16, 17].

It is therefore suggested that when tumour cells are incubated with PC, some of the missing phospholipids are restored to the cells. Trypsinization prior to PC treatment is likely to unmask a phospholipid binding site and allow PC to become an integral part of the structure of the cell wall.

The incorporation of phospholipids within the cell membrance reconstitutes the missing tissue-specific antigen to the cell surface and re-instates its immunological identity as a tissue. Tumour cells modified in this manner are then capable of eliciting a cell mediated rejection mechanism in the host.

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## SUMMARY

*A tumour-inhibitory factor was found in the post-coital uterine fluid of the rat. This factor was also present in the seminal plasma of this and other species. Chemical analysis showed it to be a phospholipid, probably glycerylphosphorylcholine or phosphorylcholine.*

*Tumours treated with extracts of seminal plasma, glycerylphosphorylcholine or phosphorylcholine, failed to grow when inoculated subcutaneously into weanling or adult rats, but gave rise to tumours when injected subcutaneously into neonates or into the brain of adults. A large proportion of neonates died of graft disease. This did not occur when the graft consisted of tumour cells only.*

*The phosphorylcholine/tumour conjugate thus produces tumours only under conditions of immunological privilege, the brain being an immunologically privileged site and neonates being in a state of immunological immaturity.*

*It is concluded that phosphorylcholine confers immunological competence to the tumour cell and converts it into a non-malignant cell in respect of its immunological reactivity.*

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# The Pattern of Cell Growth in Reticulum Cell Sarcoma and Lymphosarcoma

M. J. PECKHAM* and E. H. COOPER

*Department of Radiotherapy, Royal Marsden Hospital, Sutton, Surrey and Department of Experimental Pathology and Cancer Research, University of Leeds, Great Britain*

IT HAS been common practice to consider the results of treatment of patients suffering from reticulum cell sarcoma and lymphosarcoma together, because of the confusion which exists in the histological classification of these tumours. It is apparent, however, that the malignant lymphomas are by no means a homogeneous collection of tumours, both in terms of histological appearance and localization and natural history of the disease. Marked histiocytic phagocytosis in some cases of lymphosarcoma and reticulum cell sarcoma and the association of this activity with a poor prognosis, regardless of the extent of the disease [1, 2], suggests the tumour cell proliferation and death in the nodes may vary considerably within the same histological category. The results of previous investigations into the pattern of cell proliferation in human lymphoid malignancy have shown variation in tumour cell ploidy and labelling index between tumours of similar histological appearance [3, 4]. Further observations are reported in this paper and an attempt is made to assess the possible significance of the findings in relation to the management of patients with lymphosarcoma and reticulum cell sarcoma.

## MATERIAL AND METHODS

Fresh lymph-node material was obtained by surgical excision from 12 patients suffering from lymphosarcoma, 5 patients with mixed histiocytic–lymphocytic lymphoma, and 13 patients with reticulum cell sarcoma. Details of the preparation of specimens for tritiated thymidine ($^3$H-Tdr) labelling and DNA measurement have been described previously [3, 4]. Some specimens were labelled with $^3$H-uridine ($^3$H-UR) 4 μc/ml for 30 min.

The histological classification employed is based on that described by Rappaport [5].

## RESULTS

The results of labelling index, measurement of DNA content and the clinical features are summarized in Tables 1, 2 and 3.

1. *Reticulum cell sarcoma* (*Table* 1)

The 13 patients studied in this category can be divided into three broad groups, based on the anatomical distribution of their disease at the time of diagnosis: A. disease localized to one or two lymph areas above the diaphragm; B. generalized lymph-node disease with or without liver and spleen involvement; C. extranodal lesions.

A. The pattern of spread in the 4 patients who presented with localized disease in relation to treatment is summarized in Fig. 1. Only 1 patient remains alive and free from disease following radiotherapy. This patient received treatment not only to the involved left-cervical region, but to all lymph-node areas above the diaphragm, using the 'mantle' technique. The pattern of spread in the other 3 patients in this group suggests that a radical approach might have been more effective in eradicating the tumour. The modal DNA content of the

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* Requests for reprints to be addressed to M. J. Peckham, The Royal Marsden Hospital, Downs Road, Sutton, Surrey, Great Britain.

*Table 1. Reticulum cell sarcoma. Summary of labelling index, DNA content and clinical features*

| | Case no. | Age and sex | Sites of involvement | $^3$H-TdR labelling index | | Tumour cell modal DNA content | Treatment and response |
|---|---|---|---|---|---|---|---|
| | | | | Overall | Tumour cell | | |
| A. Patients presenting with disease localized to one or two lymph-node areas | 1 | M 56 | L. cervical | 9·4 | 28·0 | hypotetraploid | Local radiotherapy (5000 r.); no local recurrence, extension into adjacent areas |
| | 2 | M 58 | L. tonsil and L. cervical | 11·0 | 21·0 | hypotetraploid | Local radiotherapy (4000 r.); no local recurrence, adjacent extension |
| | 3 | M 49 | R. cervical | 4·0 | 32·0 | hypotetraploid | Local radiotherapy (4600 r.); extension into adjacent areas, local recurrence after 11 years |
| | 4 | M 46 | L. cervical | 5·2 | 27·4 | hypotetraploid | Radiotherapy to involved areas (4500 r.) and uninvolved (4000 r.) above diaphragm |
| B. Generalized lymph-node involvement and liver and spleen involvement | 5 | M 22 | Generalized node and bone deposits | 46·0 | 48·0 | diploid | Radiotherapy and chemotherapy; developed marrow infiltration, terminally |
| | 6 | F 72 | Generalized node and spleen | 20·0 | 24·0 | diploid | Chemotherapy, very sensitive to vincristine. Atypical monocytoid blasts in marrow |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 7 | F | 46 | General node involvement | 5·6 | 33·0 | diploid | Radiotherapy with rapid recurrence following incomplete regression (1500–2600 r.). Resistant to chemotherapy |
| | 8 | M | 46 | Generalized node and liver and spleen | 0·4 | 20·0 | hyperdiploid | Died post-operatively |
| | 9 | M | 29 | Generalized node | 2·0 | 5·4* | diploid | Chemotherapy (steroids, T.E.M., procarbazine, velbe). Radiotherapy to residual disease |
| | 10 | M | 29 | Generalized node and spleen | 7·5 | 42·0 | hyperdiploid | Palliative radiotherapy to relieve superior mediastinal obstruction; chemotherapy extremely sensitive to vincristine |
| C. Extranodal | 11 | M | 44 | L. parotid salivary gland | 25·3 | 35·0 | diploid | Rapid generalized spread following attempted radical surgery |
| | 12 | M | 79 | L. submandibular gland | 1·1 | 22·0 | diploid | No local recurrence after surgery and radiotherapy (3500 r.) with subsequent extension to L. parotid, femur, skull and abdomen |
| | 13 | M | 80 | L. buttock | 5·0 | 30·0 | diploid | Local recurrence and extension after 3000 r. |

* Arrested in DNA synthesis.

*Table 2. Summary of labelling index, DNA content and clinical features in lymphocytic lymphoma*

| Histology | Case no. | Age and sex | Sites of involvement | $^3$H-TdR labelling index | | Tumour cell modal DNA content | Treatment and response | Survival |
|---|---|---|---|---|---|---|---|---|
| | | | | Overall | Tumour cell | | | |
| Well differentiated lymphocytic lymphoma | 1 | M 50 | Generalized node involvement (leukaemic) | 0·4 | 32·0 | diploid | Well controlled on cyclophosphamide, steroids and vincristine | Alive with disease 23/12 |
| | 2 | M 6 | Generalized node involvement (leukaemic) | 0·5 | 21·0 | diploid | Initially good response to steroids, cyclophosphamide and chlorambucil | Died 14/12 |
| | 3 | M 74 | Retroperitoneal mass (leukaemic) | 0·5 | 30·0 | diploid | 3700 r. to abdominal mass with good response. Recurred 9/12, treated with chlorambucil since | Alive with disease 24/12 |
| | 4 | M 66 | Generalized node involvement, liver and spleen | 1·1 | 32·0 | diploid | Treated with cyclophosphamide and chlorambucil which temporarily controlled disease | Died 15/12 |
| Poorly differentiated lymphocytic lymphoma | 5 | F 75 | Thyroid | 13·0 | 16·3* | diploid | Local radiotherapy. Very radiosensitive with generalized disease after 6/12. Treated with steroids, vincristine and cyclophosphamide with good control | Alive without sign of disease 36/12 |
| | 6 | M 65 | Generalized predominantly extranodal disease | 14·6 | 16·8 | diploid | Initially treated with vinblastine and cyclophosphamide, then vincristine, cyclophosphamide and steroids and palliative radiotherapy | Died 32/12 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 7 | M | 26 | Retroperitoneal mass | 26·0 | 28·0 | diploid | Radiotherapy (3500 r.) to mass with subsequent extension 2/12 later | Lost to follow-up |
| 8 | F | 65 | Generalized node involvement | 5·2 | 21·7 | hyper-diploid | Effective chemotherapy and radiotherapy prevented by persistent neutropenia due to white-cell antibodies | Died 18/12 |
| 9 | M | 80 | Generalized node involvement | 12·6 | 31·0 | hyper-diploid | Dramatic regression on steroids alone, well maintained on cyclophosphamide | Alive with disease 15/12 |
| 10 | F | 40 | R. tonsil and R. cervical nodes | 3·0 | 21·0* | hyper-diploid | Local radiotherapy with recurrence and extension | Alive with disease 30/12 |
| 11 | M | 66 | General node and liver and spleen (leukaemic) | 3·5 | 26·0 | diploid | Died daunorubicin myocardopathy | Died 8/12 |
| 12 | F | 76 | General node and liver and spleen, marrow infiltrated | 0·9 | 24·0 | diploid | No response to chlorambucil and vincristine | Died 2/12 |

* $G_2$ build up.

*Table 3. Summary of labelling index, DNA content and clinical features in mixed histiocytic-lymphocytic lymphoma*

| Sites of involvement | $^3$H-TdR labelling | | Tumour cell modal DNA content | Treatment and response | Survival |
|---|---|---|---|---|---|
| | Overall | Tumour cell | | | |
| Mediastinum L. cervical (no lymphogram because of superior vena cava obstruction) | 4·6 | 11·6* | diploid | 4000 r. to involved areas with rapid dissemination not controlled by steroids, vincristine, procarbazine or T.E.M. | Died 1/12 |
| Generalized nodal and liver and spleen | 14·0 | 27·0 | diploid | Vincristine, p32, steroids, palliative, radiotherapy and cyclophosphamide | Died 3/12 |
| Generalized nodal and liver and spleen. Marrow infiltration with abnormal circulating cells | 0·8 | 9·5* | diploid | Very rapid deterioration; node haemorrhagic at autopsy | Died 4/52 |
| Generalized nodal | 8·0 | 13·0† | diploid | Radiotherapy to para-aortic, mediastinum, L. axillary and L. cervical nodes (3000–4000 r.); developed liver involvement and pleural effusion 6/12 later | Died 6/12 |
| (a) Generalized nodal with marrow infiltration | 2·8 | 26·0 | diploid | In spite of report of marrow infiltration treated with extended field radiotherapy above and below diaphragm 3000–4000 r. Then chlorambucil and vinblastine. Subsequently developed ascites | Died 17/12 |
| (b) Ascites and generalized disease | 3·8 | 5·0 | diploid | | |

* Arrested DNA synthesis.
† Build up in $G_2$.

tumour-cell populations of all 4 patients was in the hypotetraploid region, although in 1 patient a population of dividing apparently normal lymphoid cells was also identified, and the DNA contents of these cells lay within the normal range for dividing diploid cells (2C–4C), Fig. 2.

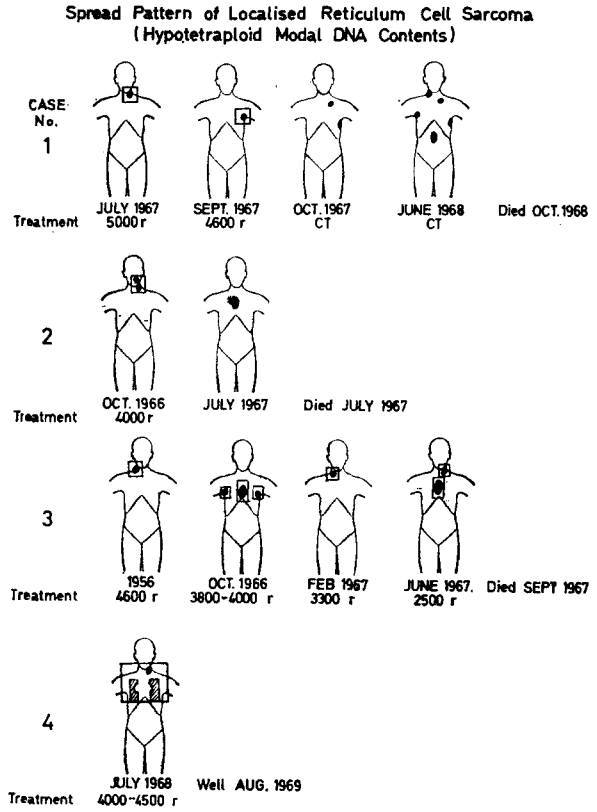


*Fig.* 1. *Pattern of spread of disease in four patients with hypotetraploid reticulum cell sarcoma.*

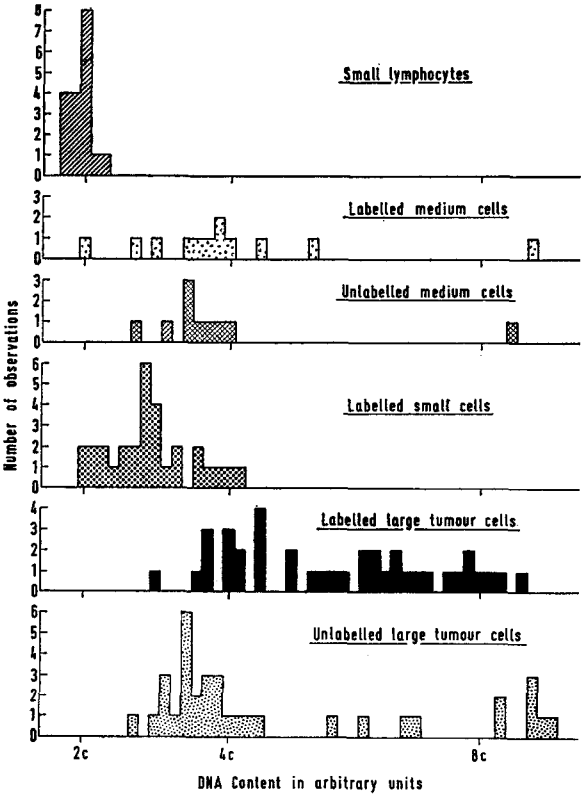


*Fig.* 2. *Distribution of DNA contents of lymph-node cells. Patient* 4, *Table* 1, *showing a hypotetraploid tumour-cell population and proliferating, small cell diploid population.*

B. The disease was confined to lymph nodes in only 2 of the 6 patients in this group. In both cases the rapid growth of the tumour prevented effective extended-field radiotherapy of lymph-node regions above and below the diaphragm. The other 4 patients had more extensive disease and were more suitable for chemotherapy. The tumours in 2 patients in this group showed marked sensitivity to vincristine with loss of node tenderness within 24 hr, followed by rapid tumour regression, Fig. 3.

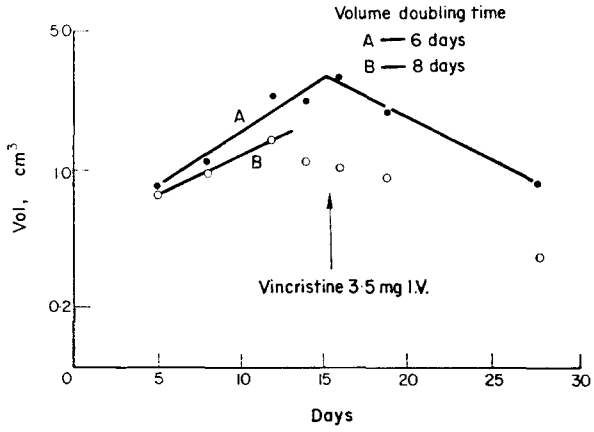


*Fig.* 3. *Change in volume of an involved opacified pelvic lymph node before and after vincristine. Patient* 10, *Table* 1.

C. Three patients presented with primary extranodal lesions. Two were treated by local radiotherapy; 1 with an inadequate dose (3000 rad) resulting in local recurrence and the second with local cure but with subsequent extension into distant extranodal sites, indicating that the irradiation of adjacent areas would not have been of value. With the exception of 1 patient, the tumour cells in patients in Groups B and C had diploid (2C) modal DNA contents.

*Labelling index in reticulum cell sarcoma*

The considerable variation in overall labelling index reflected the various extents to which proliferating cells had replaced the normal lymph-node cells. The tumour-cell labelling indices were between 20 and 40% in all but one biopsy and, in this specimen (patient no. 9), the presence of unlabelled cells with DNA contents intermediate between the 2C and 4C values were considered to be a possible indication of arrested DNA synthesis.

*Mixed histiocytic-lymphocytic lymphoma and lymphocytic lymphoma (Tables 2 and 3)*

Although there was considerable variation in both the overall and tumour-cell labelling indices, there was no obvious correlation between this parameter and the clinical course. There was evidence of a perturbation in the flow of cells through the cell cycle which resulted either in an accumulation of cells in the $G_2$ phase or a hold-up in the S phase. Three out of 5 patients with the mixed type of lymphoma exhibited this pattern. None of these patients was on therapy at the time of the study. In 1 patient, in whom the lymph-node cell proliferation pattern was normal and who subsequently developed ascites, the growth of cells in the ascitic fluid was abnormal as indicated by arrest of cells in the DNA-synthesis phase (S) and in accumulation of cells in the post-DNA synthetic pre-mitotic ($G_2$) phase (Fig. 4). This figure shows the results of

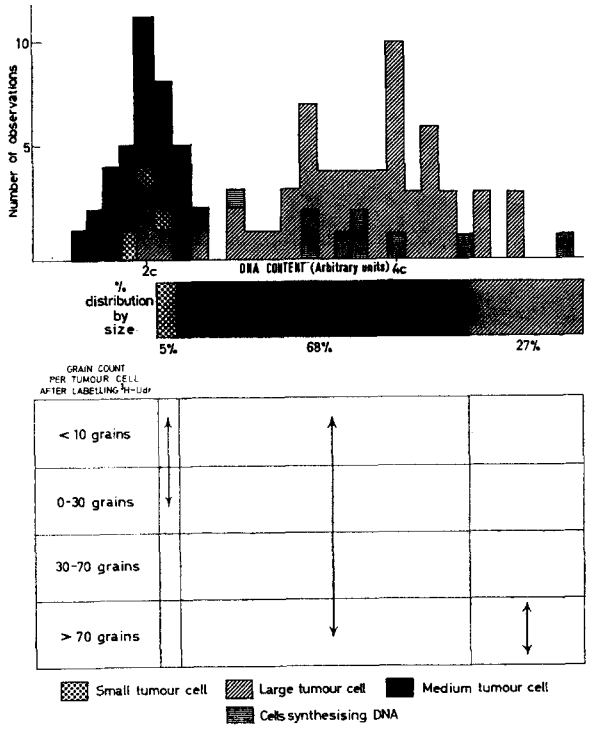


*Fig. 4. The histogram shows the distribution of DNA contents of ascites tumour cells, patient 5, Table 3. This figure also shows the range of grain counts in small, medium and large tumour cells following 30-min labelling with tritiated uridine.*

analysis of the ascitic cell population according to size and the correlation of size with nuclear DNA content and rate of RNA synthesis estimated by grain counts following pulse labelling with $^3$H-UR.

The results suggest that the small and medium-size tumour cells which contain the resting or diploid (2C) amount of DNA are heterogeneous with respect to RNA synthesis or RNA precursor pool size. The phagocytic element in the mixed lymphoid tumours was composed of non-dividing histiocytes (Fig. 5), many of which contained phagocytosed debris. This pattern was associated with a poor prognosis. All tumours in both histological categories showed diploid or near diploid modal DNA contents and these patients, as was the case in diploid reticulum cell sarcoma, tended to present with generalized disease.

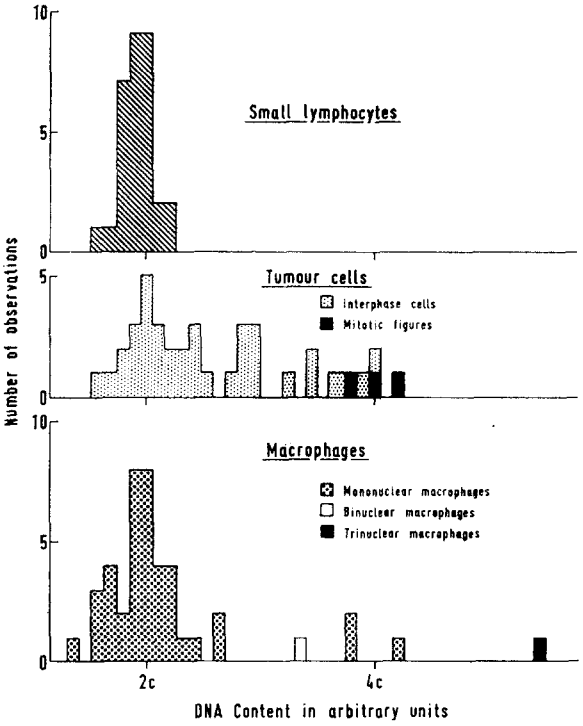


*Fig. 5. Distribution of DNA contents of small lymphocytes, tumour cells in DNA synthesis and mitosis, and macrophages, patient 2, Table 3.*

## DISCUSSION

In the present study, 4 of the 5 patients with hypo-tetraploid tumours presented with localized disease which tended, when it recurred, to spread into adjacent node areas. Diploid tumours showed a greater tendency to disseminate throughout the lymphatic system and the spread pattern in some cases suggested blood-borne metastasis. The majority presented with generalized disease. One patient, in the hypo-tetraploid group, was treated with extended-field irradiation to involved, and apparently uninvolved, areas above the diaphragm. This patient has survived so far without evidence of recurrence to 14 months and the pattern of spread in the other 3 patients suggests this type of treatment might have been advantageous.

No obvious correlation between L.I. and response to treatment of survival was observed, although it was relatively low in the mixed

histiocytic-lymphocytic tumours where the survival times were particularly short.

The pattern of proliferation, defined by the distribution of DNA contents of labelled and unlabelled cells into the various phases of interphase, allows four main categories to be defined (Fig. 6).

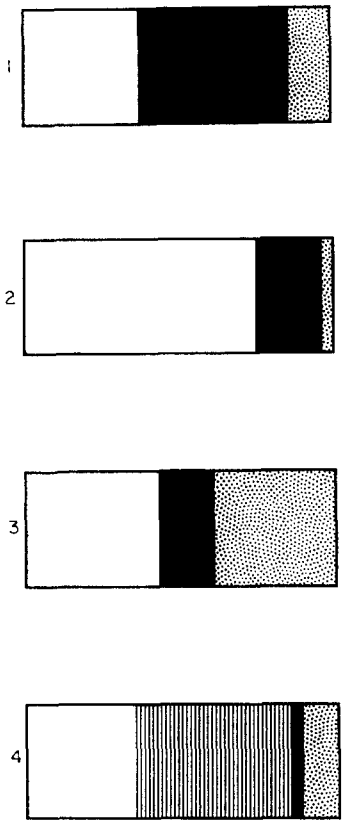


*Fig. 6. The growth patterns in human lymphoma populations. Schematic representation of the relative proportions of cells in the different phases of interphase; 1–4 are examples of the principal forms of tumour organisation.*

*Key: white: cells with resting DNA content*
*black: cells in 'S' phase*
*stippled: $G_2$ cells*
*grey: unlabelled cells with DNA contents between the resting and mitotic values.*

In the first type the proportion of cells in each of the component phases of interphase was comparable to the relative duration of the phases, estimated by *in vivo* labelling methods in human tumours [6, 7]. This suggests that the majority of cells were engaged in division. This type of pattern was encountered both in reticulum cell sarcoma and lymphosarcoma.

In the second type the labelling index of the proliferating cell type was lower and a larger proportion of cells contained the resting post-mitotic amount of DNA. This pattern was seen in poorly differentiated lymphocytic lymphoma and it is similar to that described in untreated acute leukaemia [8]. It is possible that the smaller lymphoma cells containing the post-mitotic amount of DNA represent sterile forms, although it is equally possible that this population includes cells which have only temporarily ceased to divide. Non-dividing tumour cells have been identified in solid tumours [9–11] and more recently Lala [10] has demonstrated that non-proliferating Ehrlich ascites tumour cells resume cyclic division when the total tumour-cell population is reduced in size. Recent work suggests that some non-dividing leukaemic cells in acute childhood leukaemia may re-enter the proliferative phase [12, 13], particularly when the total tumour population is reduced by chemo-therapy.

There is no evidence available to indicate that this occurs in lymphomas and sequential studies would be necessary to examine this possibility. The accumulation of small diploid tumour cells, accounting for the majority of ascitic tumour-cell population in patient 5 (Table 3), indicated that the growth fraction was low and that many cells had permanently or temporarily ceased cyclic division. The considerable variation in the grain count of the cells labelled with $^3$H-UR indicated marked heterogeneity with respect to the rate of RNA synthesis and/or RNA precursor pool size in spite of the uniformity of cell morphology and nuclear DNA content. An increased rate of RNA synthesis occurs as an early event when small lymphocytes commence cell division in response to stimulation by phytohaemagglutinin [14] and it is probable that the lymphosarcoma cells which showed high grain counts represented the true $G_1$ population while those with a low grain count could be regarded as resting cells.

The proportional distribution of proliferating cells into the $G_1$, S and $G_2$ phases indicates the fraction of the total generation time occupied by each phase. Since it is not possible to distinguish $G_1$ cells from non-proliferating but morphologically similar cells with the post-mitotic DNA content, the relative duration of $G_1$ will tend to be over-estimated in some cases.

In patients 5 and 10 (Table 2), and 4 (Table 3), the ratios of cells in S to cells in $G_2$ were 0·2, 0·4 and 0·6 respectively whereas in 6 other cases where it was possible to estimate the ratio, the proportion of cells in S to the number in $G_2$ varied from 2·5 to 6·0. This difference is due to the presence of a large proportion of $G_2$ cells in the first 3 cases and this pattern (type 3 in Fig. 6) resembles the accumulation $G_2$ cells reported in pernicious anaemia [15], that is probably the outcome of the failure of

cells to enter prophase. Clarkson *et al.* [7] reported that a proportion of mitotic figures in ascitic lymphosarcoma cells remained unlabelled after a continuous infusion of $^3$H-Tdr, suggesting that a population of cells in an extended $G_2$ phase was present. Heavily labelled mitotic figures have been observed as late as 210 hr after the label injection in human acute leukaemia which again is suggestive of a prolonged cell rest following DNA synthesis and preceding mitosis [16].

The reason for the apparent accumulation of $G_2$ cells in three tumours in the present series is unknown, they were not on chemotherapy at the time of biopsy. The disturbance to the flow of cells through interphase may be associated with an increased tumour-cell death rate similar to that described in pernicious anaemia [15]. A high rate of tumour-cell death would account for the presence of numerous reticulum cells in many of these tumours which, in some cases, have been classified as mixed histiocytic–lymphocytic lymphomas. It is perhaps more likely that the tumours are lymphocytic lymphomas infiltrated by scavenger cells consequent upon a high death rate.

The type 4 pattern (Fig. 5), patient 9 (Table 1), and 1 and 3 (Table 3) showed cells with DNA contents distributed between the 2C and 4C values, few of which had incorporated $^3$H-Tdr. There was, thus, evidence of arrested DNA synthesis. The tissue was obtained soon after removal from the patient and the availability time of the labelled precursor was identical to that employed in other preparations. The reason for the arrested synthesis is not known and it is clearly desirable to extend these observations to other tumours to see whether this pattern is common in lymphomas.

In the majority of the patients studied, the disease was already generalized by the time the diagnosis was established. These tumours showed a marked tendency to develop bone-marrow involvement and chemotherapy should obviously play a major part in their management. The use of single agents generally results in incomplete tumour regression and partial temporary control with eventual dissemination. The effect of alkylating agents in lymphocytic lymphoma and of vincristine in reticulum cell sarcoma is often dramatic. The treatment of these and other tumours might be improved in two ways: firstly, by employing a combination of drugs acting on non-dividing and dividing cells, both in mitosis and interphase and, secondly, by the use of cycle-dependent agents on a synchronized population of tumour cells. Partial synchrony has been demonstrated in acute leukaemia following cytosine arabinoside [17] but, even if synchrony could be achieved, there is insufficient data on the cell kinetics of human tumours upon which to base a rational treatment protocol.

A knowledge of the kinetic parameters of other rapidly-proliferating tissue, such as gut and marrow, is also necessary since these would be subjected to the effect of the synchronizing agent and subsequent chemotherapy. Clarkson *et al.* [7] studied the kinetics of lymphosarcoma cells in the ascitic fluid of a patient with abdominal lymphoma, but the findings in patient 5 (Table 3, Fig. 4) indicate that care should be taken in interpreting the results of kinetic studies on tumour cells which have escaped the environment of the solid-tumour mass.

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## SUMMARY

*Studies of the $^3$H-thymidine labelling index and distribution of DNA content have been made in lymph-node biopsy material from reticulum cell sarcoma and lymphosarcoma. No correlation could be established between the labelling index and the patient's response to treatment or progress. Four of 5 patients with hypotetraploid reticulum cell sarcomas presented with tumours localized to one or two lymph-node areas, and when extension of the disease occurred, this was, at least initially, to adjacent areas. Patients with tumours showing modal DNA contents in the diploid range presented either with generalized disease or disease at extranodal sites. Evidence for arrested cell proliferation in lymphoma cells is presented.*

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# The Effect of Aflatoxin B1 on RNA Synthesis and Breakdown in Normal and Regenerating Rat Liver

LUTZ WAGNER and JÜRGEN DREWS*

*Medizinische Universitätsklinik Heidelberg and SANDOZ Forschungsinstitut Wien Gesellschaft m.b.H. A-1235 Wein Brunnerstrasse 59*

## INTRODUCTION

AFLATOXIN B1, a metabolic product of aspergillus flavus, has gained wide interest as a most potent liver carcinogen. In recent years, much attention has been devoted to the possibility that the transformation of a normal cell to a cancer cell is intimately related to the formation and function of nucleic acids. It is therefore not surprising that the effects of aflatoxin on the formation of nucleic acids in mammalian cells have been the subject of a number of publications. An inhibition of DNA synthesis has been shown to occur under the influence of aflatoxin in regenerating liver [1–3] as well as in human embryonic liver cells [4–6] and human embryonic lung cells [7, 8] in culture. There have also been reports on the inhibitory effects of aflatoxin on RNA synthesis in rat liver [9, 10]. As early as 15 min after the application of a single $LD_{50}$ of aflatoxin B1, a strong inhibition of hepatic RNA synthesis was found, and could still be detected after a period of 5 days. Gelboin *et al.* [11] and Moulet and Frayssinet [12] could show that liver nuclei which had been isolated from rats treated with aflatoxin had lost part of their capacity to synthesize RNA *in vitro* as compared to liver nuclei from control rats. Nuclei from regenerating liver appeared to be more sensitive to the compound than nuclei from normal liver [12].

The experiments to be reported in this paper were designed to obtain more detailed information on the effects of a single sublethal dose of aflatoxin B1 on the synthesis and the metabolism of rat-liver nuclear RNA.

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*To whom reprint requests should be addressed.

## EXPERIMENTAL PROCEDURES

*Materials*

Aflatoxin B1 was a generous gift of Dr. G. N. Wogan (Mass. Inst. of Technology) and of Dr. D. A. van Dorp (Unilever Research Laboratories, Vlaardingen, Holland). Carrier-free $^{32}$P-orthophosphate, $^{3}$H-orotic acid, $^{14}$C-orotic acid were purchased from the Radiochemical Centre, Amersham, England. $^{3}$H-5′-adenosinetriphosphate (specific activity: 10 C/mmole) was a product of the New England Nuclear Corporation. Ethionine, actinomycin D, the four ribonucleotidetriphosphates, pronase P, and deoxyribonuclease (free of RNase) were obtained from Serva Laboratories, Heidelberg. All other compounds were chemicals of analytical purity from either Merck, A.G., Darmstadt, Serva Laboratories, Heidelberg, or Fluka Company, Buchs, Switzerland.

*Animals*

Female Wistar rats weighing 160–220 g were obtained from Brünger Animal Farms, Halle Westphalia, Germany. They were fed a standard diet (Altromin, Lage/Lippe) and tap water *ad lib.* Partial hepatectomy was carried out according to the method of Higgins and Anderson [13] under ether anesthesia. Ethionine was injected intraperitoneally as a 0·153 M solution. Aflatoxin B1 (1·0–1·25 mg/kg of body weight) was administered *i.p.* in 0·1 ml of dimethylsulfoxyde (DMSO). Actinomycin D

was also injected *i.p.* dissolved in either 0·1 ml of DMSO or in 0·5 ml of saline in a dose of 2·5 mg/kg of b.w. In order to label rat liver RNA *in vivo*, 20–50 μC of $^{3}$H-orotic acid (specific activity: 1 C/m-mole), 10 μC of $^{14}$C-orotic acid (specific activity: 50 mC/m-mole), or 0·5–1·0 mC of carrier-free $^{32}$P-orthophosphate in 0·5 ml of saline were given as an intravenous injection to each rat. Depending on the type of experiment performed, labelling periods of 40 min (pulse-labelling) or of several hours (longterm labelling) were chosen.

*Cell fractionation*

After killing the rats by decapitation the livers were quickly removed, placed into ice-cold medium A (0·25 M sucrose, 0·05 M Tris. HCl, pH 7·6, 0·003 M $CaCl_2$) and minced with scissors. The nuclei were then isolated according to the method of Di Girolamo *et al.* [14] with slight modifications [15]. The integrity of the nuclei and the purity of the nuclear preparations were regularly controlled by phase-contrast microscopy.

*Extraction of Nuclear RNA*

Total nuclear RNA was extracted using hot phenol and sodium dodecyl sulfate (SDS) [16], as described elsewhere [17]. In a number of experiments, nuclear RNA was obtained by sequential extraction of the nuclei at pH 7·6 and 0°C and subsequently at pH 8·3 and 38°C, as described by Hadjivassiliou and Brawerman [18]. All nuclear RNA fractions were treated with DNase in order to remove traces of DNA. The RNA was dissolved in 5–10 ml of aqua bidest. Tris HCl, pH 7·6, $MgCl_2$, and DNase were then added to give final concentrations of 0·1 M, 0·003 M, and 20 μg/ml respectively. The samples were incubated for 10 min at 37°C. The DNase was subsequently removed by incubating with pronase P (50 μg/ml) for another 20 min. Finally another phenol extraction was performed in the cold. The RNA was precipitated from the aqueous phase by the addition of 0·1 vol of 10% (w/v) NaCl, and 2½ volumes of absolute ethanol, collected by centrifugation and dissolved in water. It was then reprecipitated with LiCl (final concentration 2 M), washed with 66% alcohol (v/v) dissolved in water and stored at −20°C.

*Analytical procedures*

Nuclear DNA was measured as described by Burton [19]. RNA was determined according to the orcinol method [20]. Sucrose gradient sedimentation, the determination of the $^{32}$P-nucleotide composition of labelled RNA, and DNA–RNA hybridization were carried out as described previously [15, 17].

In vitro *RNA synthesis*

Determinations of nuclear polymerase activity was carried out following the procedure of Pogo *et al.* [21, 22]. The nuclei were either assayed in the presence of $MgCl_2$ alone, or with $MnCl_2$ and ammonium sulfate in addition. In the first instance, each tube contained in a final volume of 0·5 ml: 0·2 ml of nuclear suspension equivalent to 300 μg of DNA, 25 m-moles of sucrose, 5 μmoles of tris HCl, pH 8·3, 6 μmoles of β-mercapto-ethanol, 2 μmoles of $MgCl_2$, 0·1 μmoles of CTP, GTP and UTP and 6 mμmoles of $^{3}$H-ATP adjusted to a specific activity of 167 mC/m-moles.

In parallel assays $MnCl_2$ and $(NH_4)_2$. $SO_4$ were added in quantities of 3 μmoles and 20 μmoles respectively. Incubations were carried out at 37°C for various periods of time. The reactions were terminated by placing the tubes in ice and rapidly adding 0·5 ml ice-cold 10% (w/v) trichloroacetic acid (TCA) containing 0·04 M sodium pyrophosphate. 5 ml of 5% TCA, 0·02 M $Na_4P_2O_7$ were then added and the tubes were centrifuged in the cold at 1000 *g* for 10 min. The precipitates were washed twice with TCA/pyrophosphate and subsequently applied to Whatman glass-fibre filters (GF/B) by mild suction. Each filter was then rinsed with 20 ml of cold 5% TCA, dried under hot air and placed into 10 ml of scintillation fluid* for the determination of its radioactivity.

## RESULTS

*Effect of aflatoxin B1 on* in vivo *RNA synthesis in normal and regenerating liver*

Two hours and 40 min after the *i.p.* injection of aflatoxin in the specific activity of total liver nuclear RNA preparations isolated after a 40 min pulse with 0·5 or 1 mC of $^{32}$P-orthophosphate was clearly lower than the corresponding control values. This effect was observed in both, normal rats and animals with regenerating liver (Table 1). It was also found that—related to the fresh weight of liver—nuclei from aflatoxin-treated rats contained less RNA than normal rat liver nuclei.

Since this effect might have been simulated by an increased influx of water into intoxicated cells the nuclear RNA content was related to

*4 g of 2,5-diphenyloxazole and 0·1 g of 1·4- bis 2-(5-phenyl) oxazolyl benzol in 1 l toluene.

*Table* 1. *Influence of aflatoxin B*1 *on the quantity and specific activity of nuclear RNA in normal and regenerating liver*

| | $^{32}$P *i.v.* (mC) | Wet weight of liver (g) | Total radioactivity extracted (cpm) | Total RNA extracted (mg) | RNA μg/g liver (absolute) | (%) | Specific activity cpm/μg RNA (absolute) | (%) |
|---|---|---|---|---|---|---|---|---|
| Normal liver | | | | | | | | |
| Control | 1 | 17·35 | 160,574 | 2·124 | 122·4 | 100 | 75·6 | 100 |
| Aflatoxin | 1 | 16·50 | 76,051 | 1·632 | 98·9 | 81 | 46·6 | 62 |
| Control | 0·5 | 31·20 | 46,280 | 3·726 | 119·4 | 100 | 12·4 | 100 |
| Aflatoxin | 0·5 | 23·30 | 20,860 | 2·454 | 105·3 | 88 | 8·5 | 69 |
| Regenerating liver | | | | | | | | |
| Control | 1 | 10·00 | 416,328 | 0·836 | 83·6 | 100 | 498·0 | 100 |
| Aflatoxin | 1 | 12·00 | 192,100 | 0·550 | 45·8 | 55 | 349·3 | 70 |
| Control | 0·5 | 7·41 | 28,980 | 0·600 | 81·0 | 100 | 48·3 | 100 |
| Aflatoxin | 0·5 | 7·87 | 9570 | 0·288 | 36·6 | 45 | 33·3 | 69 |

Normal rats and animals with 24 hr regenerating liver received 0·25 mg of aflatoxin, dissolved in 0·1 ml of DMSO. Control animals were injected with the solvent alone. After 2 hr of exposure to the drug each rat was injected intravenously with 0·5–1·0 mC of $^{32}$P-orthophosphate. At the end of a subsequent labelling period of 40 min the animals were killed for the isolation of the nuclear RNA. The results from 8 single experiments are listed in the table. Each number represents values from at least two rats.

*Table 2. The effect of aflatoxin B1 on the RNA/DNA ratio in nuclei from normal and regenerating rat liver*

| | DNA (mg/g of liver) | | RNA (mg/mg of DNA) | | RNA/DNA (%) |
|---|---|---|---|---|---|
| Normal liver | | | | | |
| Control | 1·71 | (0·02) | 0·39 | (0·01) | 100 |
| Aflatoxin | 1·54 | (0·07) | 0·31 | (0·01) | 79 |
| Regenerating liver | | | | | |
| Control | 1·84 | (0·03) | 0·48 | (0·01) | 100 |
| Aflatoxin | 1·32 | (0·06) | 0·35 | (0·01) | 73 |

The experimental conditions were as described in the legend of Table 1. Standard deviations are given in parentheses.

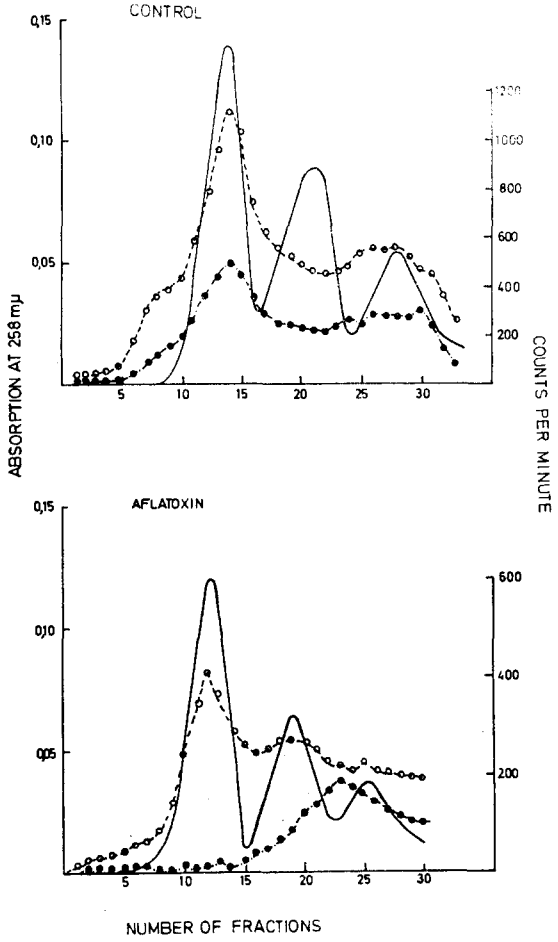


*Fig. 1. Zone-sedimentation pattern of pH 7·6 RNA extracted from liver nuclei of normal rats and animals treated with aflatoxin after double-labelling with $^{3}H$- and $^{14}C$-orotic acid. At zero time each of 4 rats received 50 μC of $^{3}H$-orotic acid by intravenous injection. After a 6-hr period 2 rats were given 0·25 mg of aflatoxin, the other 2 received the solvent DMSO only. After a subsequent 2 hr period, 10 μC of $^{14}C$-orotic acid was given to each animal. The animals were killed 40 min later for the isolation of liver nuclei and the sequential extraction of the nuclear RNA.*

*150 μg (control) and 130 μg (aflatoxin) samples of pH 7·6 RNA were layered on 5 ml linear (5–20%) sucrose gradients and centrifuged at 35,000 rpm for 4 hr in a Spinco model L preparative ultracentrifuge. The optical density of the RNA peaks was monitored by a UVI-Cord II detector attached to a recorder; fractions were collected on glass-fibre filters. The radioactivity was determined by liquid scintillation counting. Solid line: absorption at 258 nm; open circles: long-term labelling with $^{3}H$-orotic acid; closed circles: pulse-labelled material ($^{14}C$-orotic acid).*

DNA. As shown in Table 2, treatment with aflatoxin B1 is followed by a decrease in the nuclear RNA/DNA ratio.

A sequential extraction of RNA according to the method of Hadjivassiliou and Brawerman [18] allows the separation of ribosomal precursor RNA extracted at pH 7·6 from a mixture of DNA-like and ribosomal precursor RNA which is obtained at elevated temperatures and a pH of 8·3. Treatment with aflatoxin affected the labelling of both fractions; the decrease in the specific activity of the pH 7·6-fraction, however, was more pronounced than the diminution of the specific activity of the pH 8·3-fraction. This finding seemed to indicate that the formation of ribosomal precursor RNA is inhibited by aflatoxin B1 to a larger degree than the synthesis of total nuclear RNA. In order to test this possibility more closely zone sedimentation studies were performed. Rats were injected with 50 μC of $^{3}$H-orotic acid at the beginning of an experiment. After 6 hr of labelling, aflatoxin was administered to one group of animals while the other group received the solvent (0·1 ml DMSO) alone. Two hours after the administration of aflatoxin or DMSO, all rats received 10 μC of $^{14}$C-orotic acid. After a subsequent period of 40 min, the animals were killed. The radioactive nuclear RNA fractions obtained by sequential extraction at pH 7·6 and pH 8·3 were analyzed by sucrose gradient sedimentation. The sedimentation patterns of tritiated and $^{14}$C-labelled pH 7·6-RNA are depicted in Fig. 1. The absolute amount of $^{3}$H-orotic acid incorporated into pH 7·6 RNA during an 8 hr, 40 min labelling period was decreased as a result of the exposure to aflatoxin which lasted 2 hr, 40 min. Since a significant proportion of this RNA is formed before the administration of aflatoxin B1, major changes in the sedimentation pattern of this RNA which turns over at a much slower rate than pH 8·3 RNA were not expected. However, the 35 S shoulder present in the pH 7·6 control fraction was no longer detected in the corresponding fraction from treated rats and the ratio of labelled 28 S RNA to 18 S RNA had shifted significantly in favour of the latter component. The $^{14}$C-labelled material of the pH 7·6 fraction, isolated after a 40 min labelling period from control rats, sedimented with a pattern similar to that of the $^{3}$H-RNA. Treatment with aflatoxin, however, resulted in the complete stop of the synthesis of RNA heavier than 24 S. Only a heterogeneous RNA component sedimenting as a broad peak with a maximum at 10 S remained unaffected by the drug.

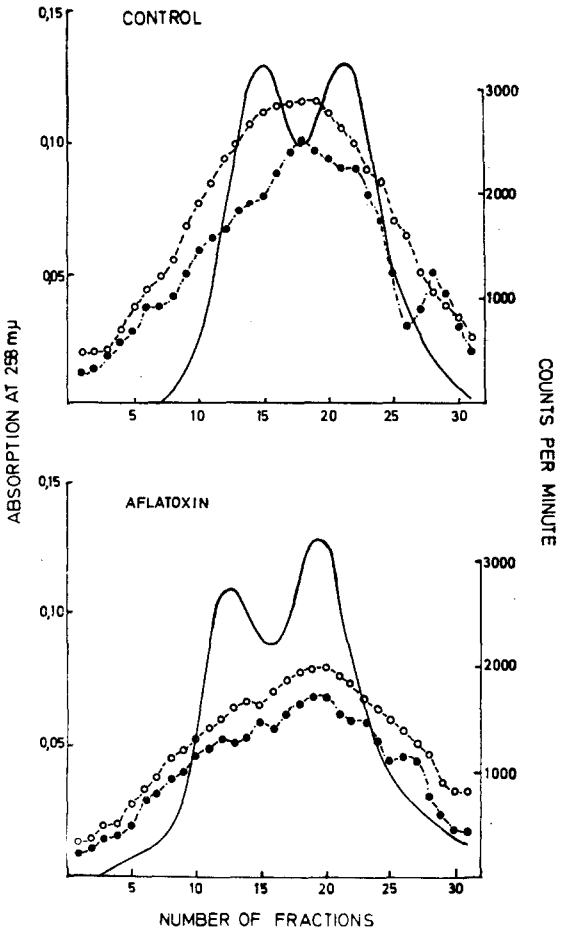


*Fig. 2. Sedimentation characteristics of pH 8·3 nuclear RNA from normal and aflatoxin treated rats. Experimental details are as described in Fig. 1. Solid line: absorption at 258 nm; open circles: RNA labelled for 8 hr 40 min; closed circles: pulse-labelled RNA.*

The $^{3}$H- as well as the $^{14}$C-labelled component of the pH 8·3 fraction sedimented as heterogeneous peaks between 28 and 18 S (Fig. 2). Although the total amounts of $^{3}$H- and $^{14}$C-radioactivity incorporated into the pH 8·3 fraction showed a decrease of about 30% as a response to treatment with aflatoxin B1 no distinct alterations in the sedimentation patterns were observed as compared to the sedimentation behaviour of pH 8·3 RNA from normal rats. These results demonstrate that 2 hr, 40 min after treatment with aflatoxin B1 the formation of ribosomal precursor RNA is significantly inhibited.

*Determination of $^{32}$P-Nucleotide composition*

As illustrated in Table 3, treatment with aflatoxin leads to a relative decrease of pulse-labelled total nuclear liver RNA in $^{32}$P-labelled CMP and GMP and a relative rise in AMP and UMP. This effect is more pronounced in 24 hr-regenerating liver than in normal liver. Nuclear RNA fractions from

*Table 3. The influence of aflatoxin B1 on the $^{32}P$-nucleotide composition of nuclear RNA from normal and regenerating liver*

| | Number of preparations | Analyses | Total radioactivity (cpm) | AMP | UMP | CMP | GMP | AMP+UMP/CMP+GMP |
|---|---|---|---|---|---|---|---|---|
| Normal liver | | | | | | | | |
| Control | 3 | 8 | 200,000 | 25·3 (0·7) | 22·7 (1·0) | 27·4 (0·9) | 24·6 (0·6) | 0·92 (0·02) |
| Aflatoxin | 3 | 6 | 95,000 | 26·5 (1·0) | 24·4 (1·3) | 26·0 (1·4) | 23·1 (1·4) | 1·04 (0·02) |
| Regenerating liver | | | | | | | | |
| Control | 2 | 7 | 380,000 | 22·9 (0·6) | 21·5 (1·1) | 28·4 (0·6) | 27·2 (0·7) | 0·79 (0·03) |
| Aflatoxin | 2 | 8 | 150,000 | 28·0 (1·1) | 22·9 (0·7) | 24·8 (1·1) | 24·3 (0·9) | 1·03 (0·06) |

Rats with normal or 24-hr regenerating liver were injected with 0·25 mg of aflatoxin in DMSO or with DMSO only at zero time. After a 2-hr period, the animals received intravenous injections of 0·5 or 1·0 mC of $^{32}P$-orthophosphate. The rats were killed at the end of a 40-min labelling period. Nuclear RNA was extracted in one step, purified and subjected to alkaline hydrolysis. The 2′3′-ribonucleotides were separated by high voltage electrophoresis.

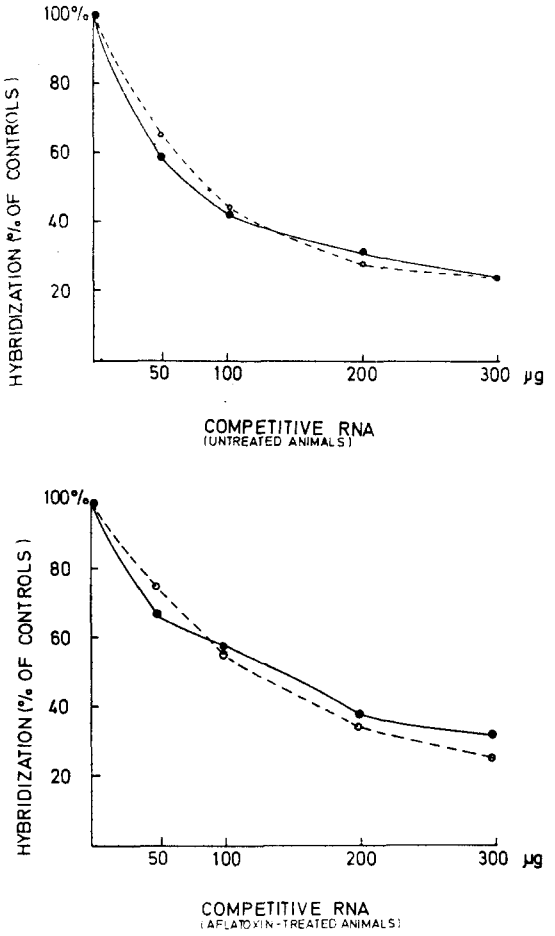


*Fig. 3. DNA–RNA hybridization–competition experiment with pH 8·3 nuclear RNA from normal rats and from rats treated with aflatoxin. Twenty µg of $^{32}$P-RNA from untreated and 20 µg of $^{3}$H-RNA from aflatoxin treated rats were incubated at 68°C with 20 µg of heat denatured rat liver DNA in the presence of increasing concentrations of competing unlabelled pH 8·3 nuclear RNA from either treated or untreated rats. The reactions were carried out in 4 × SSC (SSC = 0·15 NaCl and 0·015 of sodium citrate) for 20 hr.*
*Specific activities: $^{32}$P-RNA: 700 cpm/µg of RNA*
*$^{3}$H-RNA: 246 cpm/µg of RNA.*
*Blanks (incubated without DNA): 0·3 cpm($^{3}$H) and 1·2 cpm($^{32}$P).*
*100% values (no competing RNA): 48 cpm($^{3}$H) and 64 cpm($^{32}$P).*
*Broken line: RNA from normal rats.*
*Solid line: RNA from aflatoxin-treated rats.*

normal and from regenerating liver differ with respect to their relative proportions of DNA-like RNA and ribosomal RNA which is reflected by the lower AMP + UMP/CMP + GMP ratio in RNA from regenerating liver. This difference, which is well documented by experimental results from a number of laboratories [15, 23], is eliminated by aflatoxin B1. It is concluded from this result that the preferential formation of ribosomal precursor RNA which is a characteristic biochemical feature of regenerating liver is suppressed by aflatoxin to a considerable degree.

*Influence of aflatoxin on rapidly hybridizable RNA*

In order to test the possibility that aflatoxin, in addition to inhibiting the formation of ribosomal precursor RNA and RNA synthesis in general, induces changes in the population of rapidly hybridizable RNA in liver cells, DNA–RNA hybridization experiments were carried out. Twenty µg of $^{32}$P-labelled nuclear pH 8·3 RNA from untreated rats and 20 µg of $^{3}$H-labelled pH 8·3 RNA from rats which had received aflatoxin B1 were hybridized with 20 µg of heat-denatured liver DNA in the presence of increasing concentrations of labelled pH 8·3 RNA from either normal or aflatoxin-treated rats. As is shown in Fig. 3 no significant differences in the resulting competition curves were observed.

In vitro *RNA synthesis*

Since sufficient evidence had been obtained

*Table 4. The effect of aflatoxin B1 on RNA synthesis in ioslated rat liver nuclei*

| | Number of experiments | Omitted | Cpm/mg DNA | | Incorporation (%) |
|---|---|---|---|---|---|
| Control | | | | | |
| $Mg^{++}$ | 2 | — | 1606 | (170) | 100 |
| $Mn^{++}$ | 3 | — | 3120 | (352) | 100 |
| Aflatoxin | | | | | |
| $Mg^{++}$ | 2 | — | 1054 | (130) | 66 |
| $Mn^{++}$ | 3 | — | 2426 | (280) | 78 |
| Control | | | | | |
| $Mg^{++}$ | 1 | CTP, GTP, UTP | 89 | | 5 |
| $Mn^{++}$ | 1 | CTP, GTP, UTP | 156 | | 5 |
| Aflatoxin | | | | | |
| $Mg^{++}$ | 1 | CTP, GTP, UTP | 80 | | 6 |
| $Mn^{++}$ | 1 | CTP, GTP, UTP | 139 | | 6 |

Nuclei were isolated from control rats and from animals which had received 0·25 mg of aflatoxin B1 2 hr previously. The nuclei were incubated either in the presence of $MgCl_2$ alone or with $MnCl_2$ and ammonium sulfate in addition. The reactions were allowed to proceed for 6 min. The number of individual experiments carried out is listed in the table. Each experiment was done in duplicate or in triplicate assays. Standard deviations in parentheses.

in the experiments described above documenting the inhibitory effect of aflatoxin B1 on the formation of ribosomal precursor RNA *in vivo* it seemed warranted to study this effect *in vitro*. Pogo *et al.* [22] and Tata and Widnell [24] have demonstrated that isolated liver nuclei when incubated in the presence of low concentrations of $Mg^{++}$ synthesized a product resembling ribosomal precursor RNA in base composition while a DNA-like product was formed in the presence of $Mn^{++}$ and a high concentration of either $(NH_4)_2SO_4$ or NaCl. Liver nuclei from rats which had received aflatoxin 2 hr 40 min prior to being sacrificed displayed less capacity to synthesize RNA *in vitro* than nuclei from control animals. This inhibitory effect was more pronounced when the nuclei were incubated in the presence of $Mg^{++}$ although a significant inhibition was also observed in the $Mn^{++}$/ammonium sulfate activated reaction. Although this result does not exclude an inhibitory effect of aflatoxin on RNA species other than ribosomal precursor RNA it is compatible with the concept that the formation of ribosomal RNA is more sensitive to the action of the drug than the synthesis of DNA-like RNA species.

*The effects of aflatoxin, ethionine and actinomycin D on the breakdown of labelled RNA in liver nuclei*

As mentioned above, aflatoxin, administered *in vivo*, leads to a decrease in the RNA content of rat liver nuclei. In addition to an impairment of RNA synthesis, this loss of RNA could be the consequence of a more rapid breakdown of newly synthesized RNA. In order to test the latter possibility the effect of aflatoxin on the labelling of nuclear rat liver RNA was compared to the corresponding effects of actinomycin D and ethionine. Stewart and Farber [25] have recently demonstrated that ethionine, while inhibiting RNA synthesis, does not accelerate the breakdown of preformed RNA. On the contrary, actinomycin D does cause the loss of rapidly labelled RNA moieties in addition to its well-known effect on the formation of RNA. In a preliminary experiment, the relationship between the dose of ethionine administered to rats of 200 g b.w. and the resulting inhibition of the labelling of nuclear liver RNA had to be studied. As depicted in Fig. 4, an almost complete inhibition of the incorporation of labelled precursor is produced by a dose of approximately 1 g ethionine per kg of body weight after a 4-hr time interval. This finding is in accord with the results of other authors [26].

In an attempt to study the effect of aflatoxin on the breakdown of nuclear RNA, 8 rats were injected with $^3$H-orotic acid. After a 2-hr period, 2 rats were killed for the determination of the specific activity of the nuclear liver RNA. The remaining 16 animals were divided into 2 groups, one of which received 200 mg of ethionine, while the other group was injected with 8 ml of saline only. At the end of a subsequent 4-hr period, 2 animals of each group were killed. The remaining 6 rats of each group were again divided into three sets of animals with 2 rats in each. One set of each group

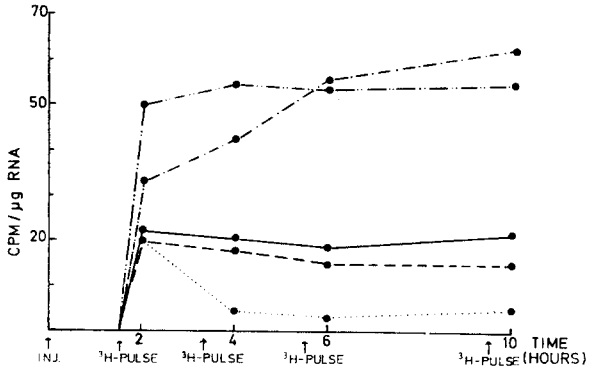


*Fig. 4. Five groups with 8 rats in each were treated as follows: animals in group 1 and 2 received* i.p. *injections of 8 ml and 3 ml saline respectively. Ethionine was given to group 3 (solid line) at 75 mg per animal. Group 4 (dashed line) was injected with 125 mg per rat, and group 5 received 200 mg. 2 animals of each group were killed after 2, 4, 6 and 24 hr. Forty minutes prior to being sacrificed each animal received an intravenous injection of 50 µC orotic acid. Total liver nuclear RNA was extracted for the determination of the specific activity. The figure illustrates results from 2 experiments; each point represents an average value from 4 rats.*

(NaCl or ethionine) received DMSO alone, the next set was injected with aflatoxin, while actinomycin D was administered to the last set. The radioactivity of the liver nuclear RNA was then measured after another period of 2 hr. This experiment was performed four times with virtually identical results. As illustrated in Fig. 5 the specific activity of nuclear RNA declines significantly between the second and the sixth hour of the experiment as a consequence of the administration of ethionine. The additional application of aflatoxin to this group after 6 hr did not accelerate the loss of radioactivity from nuclear RNA as compared to animals injected with DMSO alone. Actinomycin D, on the other hand, induced a loss of radioactivity, additional to that produced by ethionine alone or by the successive application of ethionine and aflatoxin B1. Four hours after the injection of ethionine RNA synthesis is already maximally inhibited (Fig. 4) [26]. Therefore, it appears justified to attribute the additional fall in the radioactivity of nuclear RNA observed after injection of actinomycin D to an accelerated breakdown of previously labelled RNA species. Aflatoxin does not produce this catabolic effect.

## DISCUSSION

Aflatoxin B1, administered to rats in a sublethal dose of 1 mg/kg of body weight, leads to an impairment of RNA synthesis in liver nuclei within 2 hr after its application. The formation of ribosomal precursor RNA is inhibited by this compound more severely than the formation of nonribosomal nuclear RNA species.

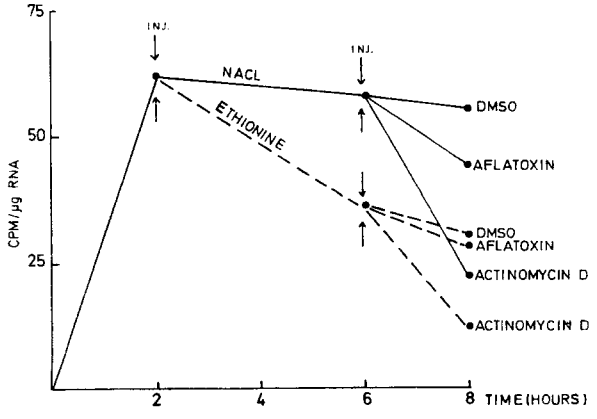


*Fig. 5. The effect of pretreatment of rats with ethionine on the specific activity of total nuclear RNA from liver as a function of time after administration of 25 µC of ³H-orotic acid at zero time, ethionine or NaCl at 2 hr, and DMSO, aflatoxin or actinomycin D at 6 hr. Experimental details are given in the text. Each point represents values from 8 rats.*

Our results are in general agreement with the findings of Lafarge *et al.* [27] who studied the inhibition of RNA synthesis exerted by aflatoxin in rat liver nuclei and nucleoli and found the formation of nucleolar RNA to be more severely impaired by the drug than total nuclear RNA synthesis. Electron microscopic studies have shown that doses of aflatoxin comparable to those used in this investigation cause morphologic alterations in the structure of liver nucleoli which become manifest after the inhibition of RNA synthesis is already established. These alterations have been reported to be reversible after 24 hr [28] or to persist as long as 72 hr [29]. In view of the findings described in the preceding section one is inclined to regard the changes in nucleolar structures as morphologic equivalents of the preferential inhibition of the synthesis of ribosomal RNA. It is difficult however, to interpret these phenomena in the context of carcinogenesis. Whether applied chronically in small doses or given as a relatively high single dose, aflatoxin can produce hepatomas and other malignancies [30–35]. Destructive changes of the nucleoli, however, are only observed when the compound is given in acutely toxic doses. When rats were maintained on diets, containing 1–2ppm* [29] or 0·015 ppm [36] of aflatoxin no early morphologic changes were detected in the nucleoli. Nucleolar enlargements were found only after the animals had been on a diet containing aflatoxin for 33 weeks. Nevertheless 80% of the rats had developed hepatomas 52 weeks after the institution of an aflatoxin-containing diet [29].

Thus, an early inhibition of the synthesis of ribosomal RNA does not appear to be a meaningful link in the chain of biochemical events by which a normal liver cell is transformed into a hepatoma cell. At the present time, it appears more reasonable to regard the early effects of aflatoxin on the formation of ribosomal RNA as an acute toxic manifestation.

On the basis of comparative studies on the mechanism of action of actinomycin D and ethionine Stewart and Farber [25] have suggested that compounds which inhibit RNA synthesis by binding to the DNA template also enhance the turnover of RNA while drugs which interfere with RNA synthesis by a different mechanism do not exert such additional effects. Although aflatoxin belongs to the category of inhibitors of RNA synthesis which possess a high affinity for DNA no experimental evidence was found in this study which would document such an interference of aflatoxin with the metabolism of nuclear RNA. Therefore, the capacity of drugs to associate with DNA does not appear to justify any conclusion regarding their effect on the breakdown of preformed RNA.

**Acknowledgements**—We are indebted to Miss Sabine Johne and to Mrs. Sabine Mocikat for competent technical assistance.

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*ppm = parts per million.

## SUMMARY

*The acute effects of a single sublethal dose of aflatoxin B1 on the metabolism of nuclear RNA in normal and regenerating rat liver were investigated.*

(1) *Two-and-a-half hours after the intraperitoneal injection of aflatoxin B1 into rats in doses between 1·0 and 1·25 mg/kg of body weight a 30–50% decrease in the incorporation of labelled RNA precursors into rat liver nuclear RNA is observed. Aflatoxin also induces a fall in the RNA/DNA ratio of rat liver nuclei.*

(2) *As judged by the sedimentation profiles of labelled nuclear RNA, obtained by sequential extraction, treatment with aflatoxin impairs the formation of 35 S ribosomal precursor RNA and 28 S RNA.*

(3) *The administration of aflatoxin is also followed by a shift in the $^{32}$P-nucleotide composition of total nuclear RNA extracted after* in vivo *pulse-labelling with $^{32}$P-orthophosphate to more DNA-like values. The rise in the AMP+UMP/CMP+GMP ratio of $^{32}$P-labelled RNA which follows the application of aflatoxin B1 is more marked in 24-hr regenerating liver than in normal liver.*

(4) *These* in vivo *results are paralleled by findings from* in vitro *experiments: nuclei, isolated from aflatoxin-treated rats display less capacity to synthesize RNA* in vitro *than control nuclei. This inhibition is maximal when the nuclei are maintained under conditions which are known to favour the formation of an RNA product resembling ribosomal RNA in base composition.*

(5) *Aflatoxin does not induce alterations in the population of rapidly hybridizable RNA species which are detectable by DNA-RNA hybridization experiments.*

(6) *No experimental evidence for an acceleration of the breakdown of preformed hepatic nuclear RNA was found after treatment with aflatoxin B1 while such an effect could be shown to occur after the administration of actinomycin D.*

*These results which seem to indicate that aflatoxin B1 exerts a preferential inhibition on the formation of ribosomal RNA are discussed in context with the well-known hepatotoxic and carcinogenic properties of aflatoxin B1.*

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# Fine Structure of Murine Pulmonary Adenocarcinomata Induced by Treatment with 20-Methylcholanthrene in Organ Culture*

BOJAN FLAKS† and ANTONIA FLAKS

*Department of Experimental Pathology and Cancer Research, The Medical School, University of Leeds, Leeds, England*

## INTRODUCTION

IN A PREVIOUS paper [1], a description was presented of the fine structure of murine pulmonary adenomata, which had been induced by the exposure of lung tissue explants to 20-methylcholanthrene in organ culture, followed by their subcutaneous implantation into the flanks of isologous host mice. These lesions appeared to be derived from type II alveolar cells and resembled, in many respects, those which arise spontaneously or have been induced *in vivo* in the mouse [2–5]. They were all non-invasive tumours of microscopic size. The intention of the present investigation was to examine the fine structural organization of primary murine pulmonary adenocarcinomata induced by an *in vitro* procedure, identical to that which was used in the previous studies [1, 6, 7]. This would permit a direct comparison of the ultrastructure of these carcinomata with that of the previously studied adenomata. It would thus provide a system for the study of morphological changes associated with the acquisition of an invasive character by murine pulmonary tumours.

## MATERIAL AND METHODS

Pure-line BALB/c mice were bred in this laboratory by intensive brother–sister mating. These were used both to provide lung tissue explants and as hosts for implantations. The techniques of organ culture and implantation used here have been described previously [6, 7]. Explants of lung tissue, not more than 1 mm thick and up to $2 \times 3$ mm in area, were removed from young adult mice. The explants were maintained in organ culture for 1–6 days in a culture medium which contained 20-methylcholanthrene at a concentration of 4 μg/ml. To minimize the amount of free 20-methylcholanthrene introduced into host animals during implantation, the explants were transferred into the basal medium, in which they were maintained for a further day. They were then implanted subcutaneously into the flanks of the isologous host mice.

Beginning at three months after implantation, the host animals were killed at monthly intervals during a period of nine months. Their implants were removed and processed for electron microscopy. If the implants were large enough at autopsy, portions of them were used for examination by routine optical microscopy, and portions were also implanted subcutaneously into the flanks of isologous mice to permit an assessment of their transplantability.

Periods of exposure to the carcinogen of 1–6 days were used since they had been shown [7] to give higher yields of tumours in treated lung implants than a longer period [6] which resulted in more necrosis. Since adenocarcinomata form a small proportion of the total number of tumours obtained by this technique [7], a high tumour yield was neces-

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†Present address: Department of Pathology, The Medical School, University of Bristol, Bristol, England.

sary to provide sufficient material for the present experiment.

For electron microscopic studies the tissue was immediately placed in ice-cold cacodylate-buffered 5% glutaraldehyde, pH 7·2 [8, 9] and finely minced. Fixation was continued for a total of 4 hr, after which time the tissue was washed in cacodylate-buffered 0·25 M sucrose for 16 hr at 0°–4°C. It was then postfixed for 2 hr at 0°–4°C in phosphate-buffered 1% osmium tetroxide [10]. The fixed tissue was rapidly dehydrated in ethanol and embedded in Epon 812 [11]. Thin sections were cut on Ge-Fe-Ri diamond knives, using a Sorvall MT-1 Porter–Blum ultramicrotome. The sections were mounted on naked copper grids, stained with lead tartrate [12] and examined in an RCA EMU-3G electron microscope at 100 kV.

Semi-thin sections, approximately 0·25 to 0·5 μ in thickness, were also prepared from the Epon-embedded material. These were stained with 1% methylene blue in 1% borax and examined by optical microscopy to aid the selection of suitable specimens for electron microscopy. Preference was given to lesions which were as small as possible, while still showing clear evidence of invasiveness, with infiltration of the surrounding tissues.

Although it was possible for both adenomata and adenocarcinomata to be present in the same implant, the adenocarcinomata were large enough to be removed and samples taken without the risk of including adenomatous tissue in the sample.

## OBSERVATIONS

The histological appearance of the tumours selected for electron microscopic studies was that of small invasive adenocarcinomata (Fig. 1). Additional evidence of the malignant nature of these tumours was afforded by their behaviour following subcutaneous transplantation into fresh hosts. The transplanted tumours grew rapidly in serial passages, invaded the surrounding tissues and, occasionally, metastasized to the liver. The tumour cells were variable in size and irregularly polygonal in outline. They possessed rounded to oval nuclei and were rather loosely packed. Their cytoplasm contained variable numbers of dense granules which were clearly visible in semi-thin epon sections. Numerous strands of connective tissue were present between the groups of tumour cells. Small cystic spaces were observed in many of these tumours (Fig. 2). These were lined by neoplastic epithelium and contained masses of dense, flocculent material within their lumina. The lining cells contained dense cytoplasmic granules and in other respects resembled the greater bulk of the tumour cells.

*Electron microscopy*

The variations in the ultrastructural organization of the cells of these adenocarcinomata were considerably greater than those observed in the previously studied adenomata [1]. However, it was also apparent from the electron microscopical observations that a single basic cell type was present, which generally bore a close resemblance to the cells which were characteristic of these adenomata.

The tumour cells were of epithelial type, exhibiting a well-marked polarity. They were often arranged in a random manner, although rows of cells, acinar-like structures and small cysts were fairly common. The cells possessed abundant cytoplasm and large, oval, indented nuclei.

The tumour-cell cytoplasm characteristically contained dense, membrane-bounded inclusion bodies. Their contents consisted of highly osmiophilic, close-packed parallel or concentric lamellae. The relative abundance of these inclusions varied, between wide limits, from cell to cell. In a few cells they formed quantitatively a major component of the cytoplasm while in other cases they were almost entirely absent. The possession of a cytoplasm lacking in lamellar inclusions appeared to be associated with other ultrastructural characteristics, which are to be discussed below. The inclusion bodies appeared to be secreted from the apical surfaces of the tumour cells. Material which was apparently identical in ultrastructure to the contents of these bodies was frequently observed in the "acinar" spaces near the apices of the tumour cells and formed large masses within the cystic spaces lined by tumour epithelium. The latter corresponded to the dark flocculent masses observed in semi-thin sections by light microscopy. The free material sometimes became more loosely packed and formed bizarre grid-like patterns in the extracellular spaces.

Abundant free ribosomes were present in the tumour-cell cytoplasm, commonly arranged in rosette-like or spiral polysomal aggregates. However, in some cells free ribosomes were especially numerous and were then usually present as single particles. In these cells the moderately abundant granular endoplasmic reticulum possessed numerous attached ribosomes which also appeared to be unaggregated.

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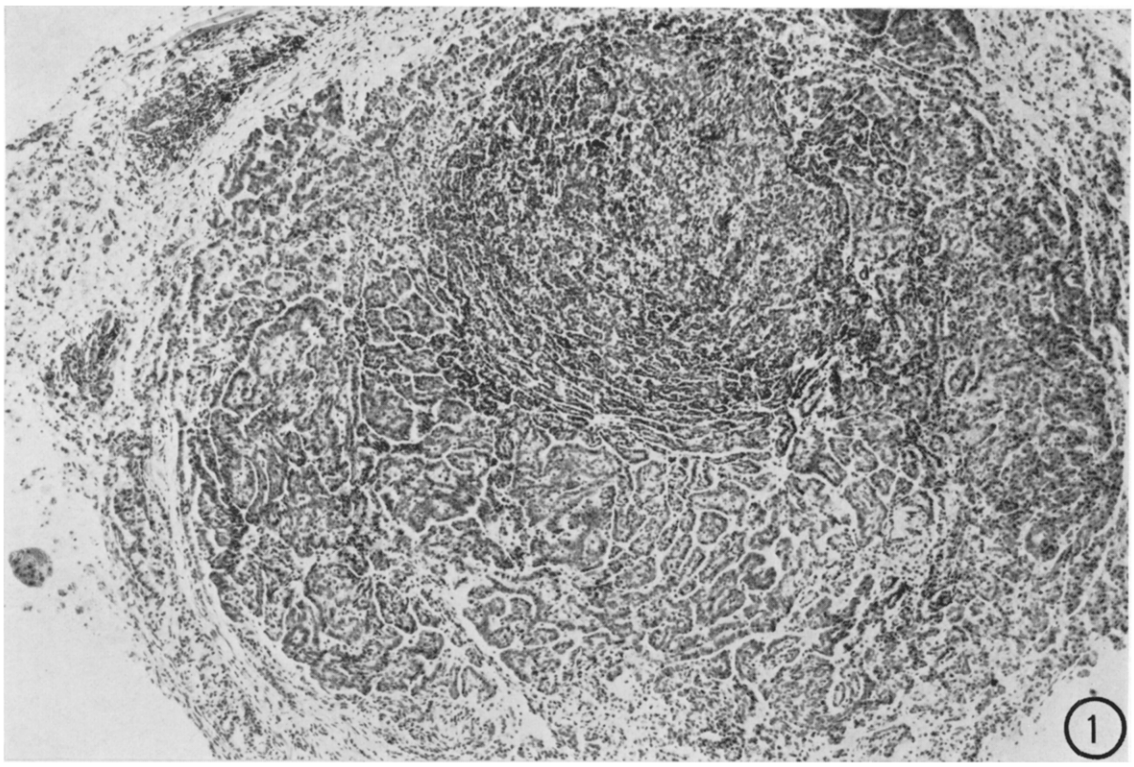

*Fig.* 1. *Low power photomicrograph of a whole carcinogen-treated implant, showing the presence of tumours.* $\times 78$.

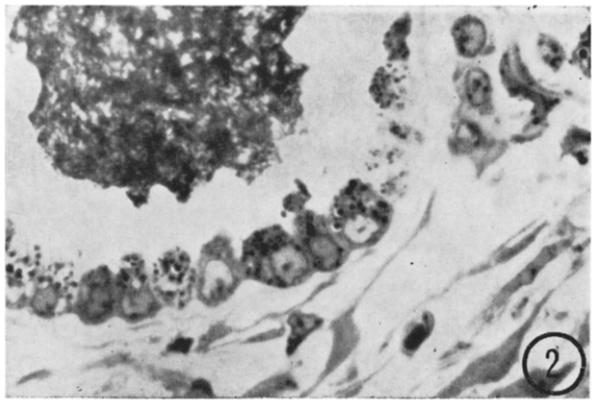

*Fig.* 2. *Cystic space in pulmonary adenocarcinoma, showing dense flocculent mass in lumen. The lining tumour-cells possess large dense cytoplasmic granules. Epon section, methylene blue staining.* $\times 1400$.

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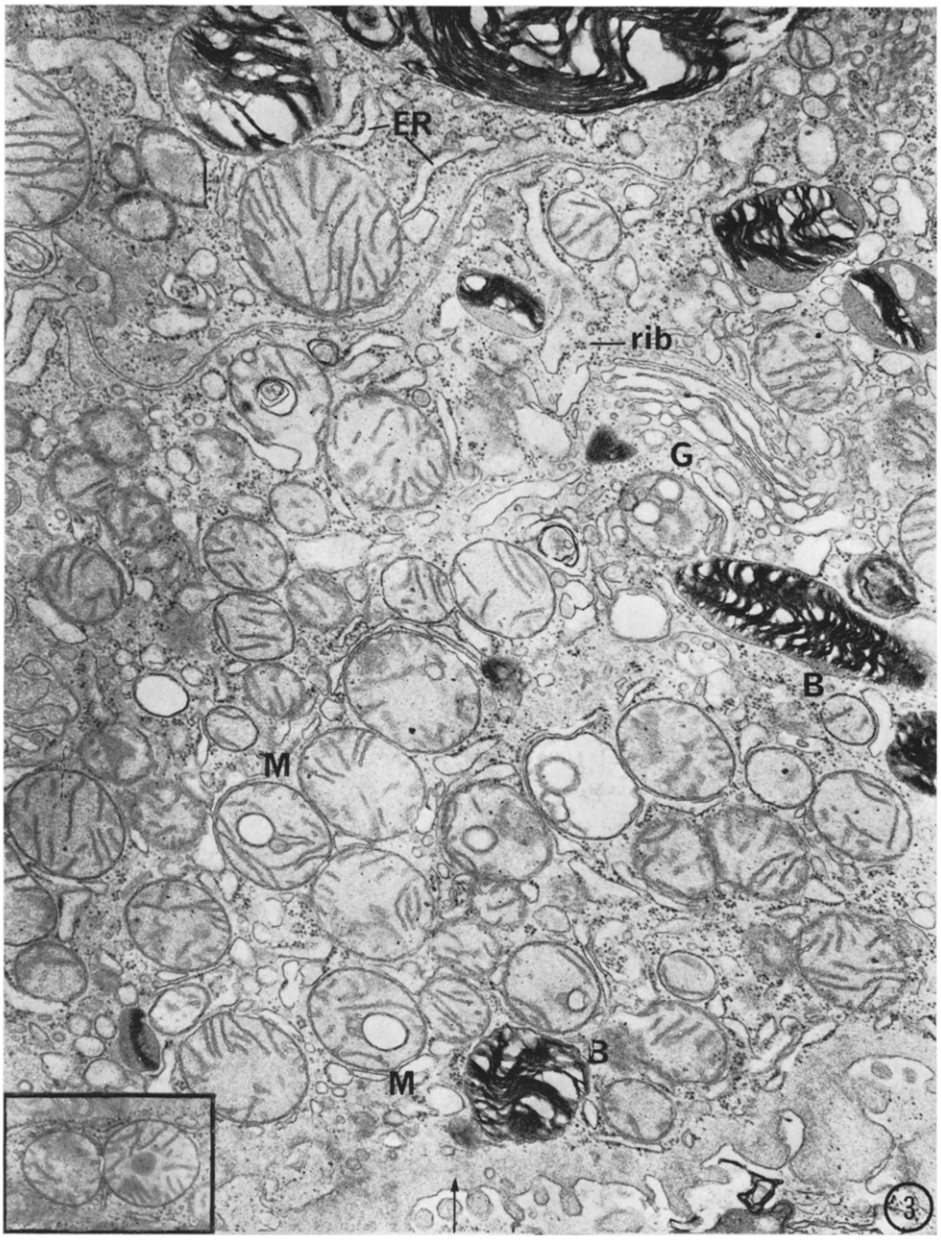


*Fig. 3. Adenocarcinoma-cell cytoplasm. The mitochondria (M) frequently contain small circular vesicular spaces. A Golgi zone is present (G) as well as a number of lamellar inclusion bodies (B) and short irregular cisternae of the granular endoplasmic reticulum (ER). Ribosomes are arranged in rosettes or spirals (rib). Note the band of marginal cytoplasm (arrows).* ×26,000. Inset: "*Division*" *form of mitochondrion.* ×19,000.

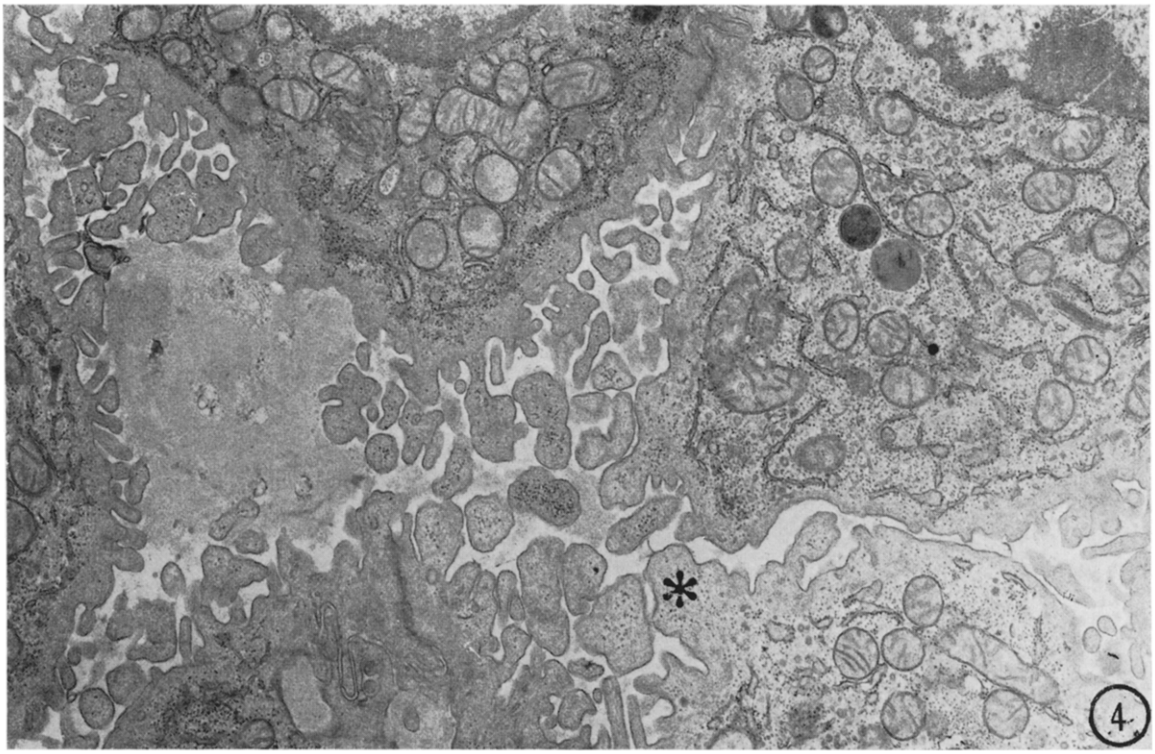


*Fig.* 4. *Adenocarcinoma cells showing loss of aggregation of ribosomes and scarcity of characteristic inclusion bodies. Irregular blebbings of the apical surface are present* (*). ×14,000.

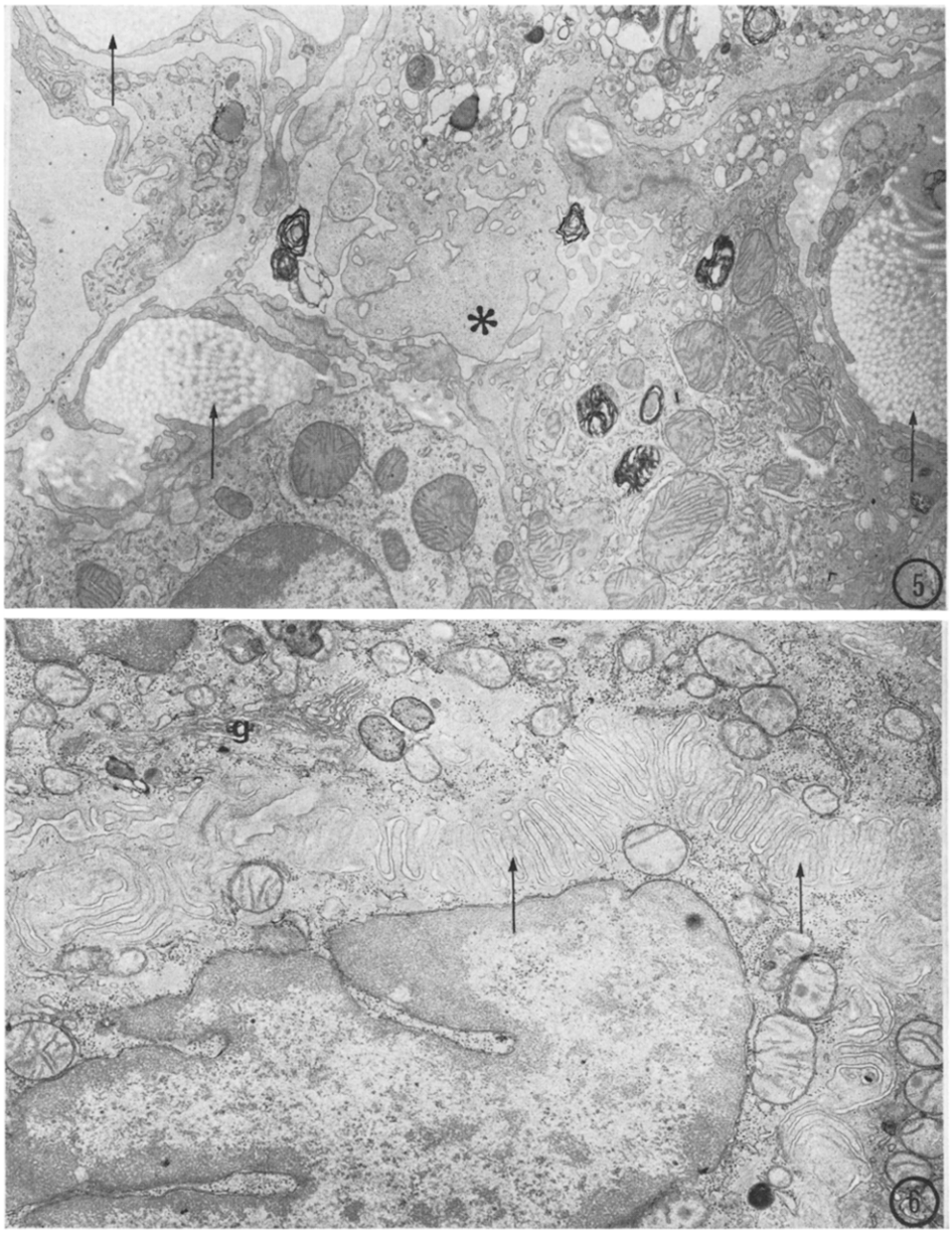


*Fig.* 5. *A large irregular cytoplasmic protusion is shown* (*), *extending into an "acinar" space. Abundant collagen is present at the basal aspects of tumour cells* (*arrows*). × 9500.

*Fig.* 6. *Electron micrograph showing interdigitating lateral cell surfaces* (*arrows*), *covered by long microvillous projections. A small Golgi zone is present* (*G*). × 16,500.

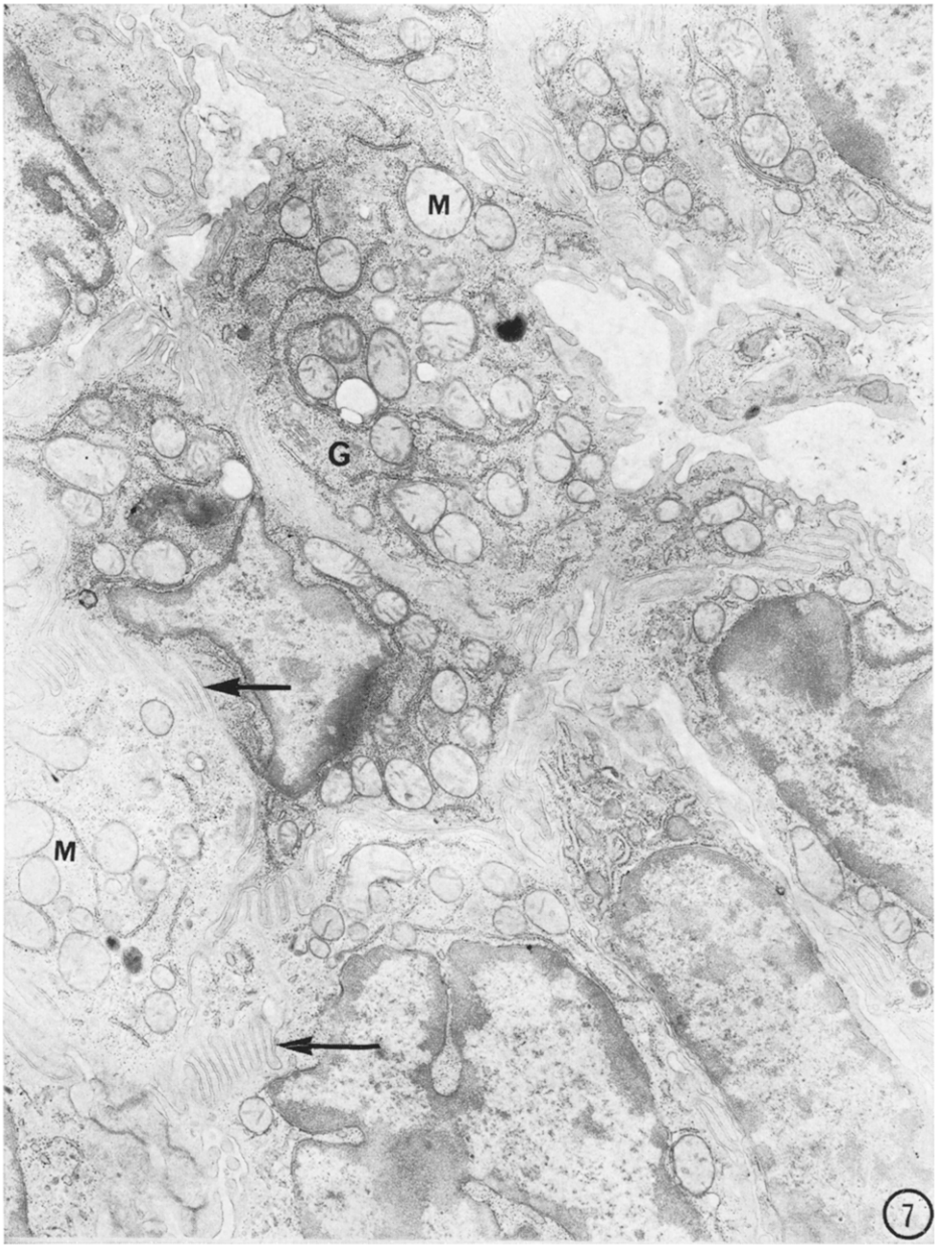


*Fig. 7. A group of relatively undifferentiated adenocarcinoma cells is shown. Many mitochondria contain few cristae (M), the Golgi zones are relatively small (G) and the ribosomes are present as single particles. The lateral cell surfaces bear long interdigitating processes (arrows). There is a scarcity of inclusion bodies* $\times$ 12,500.

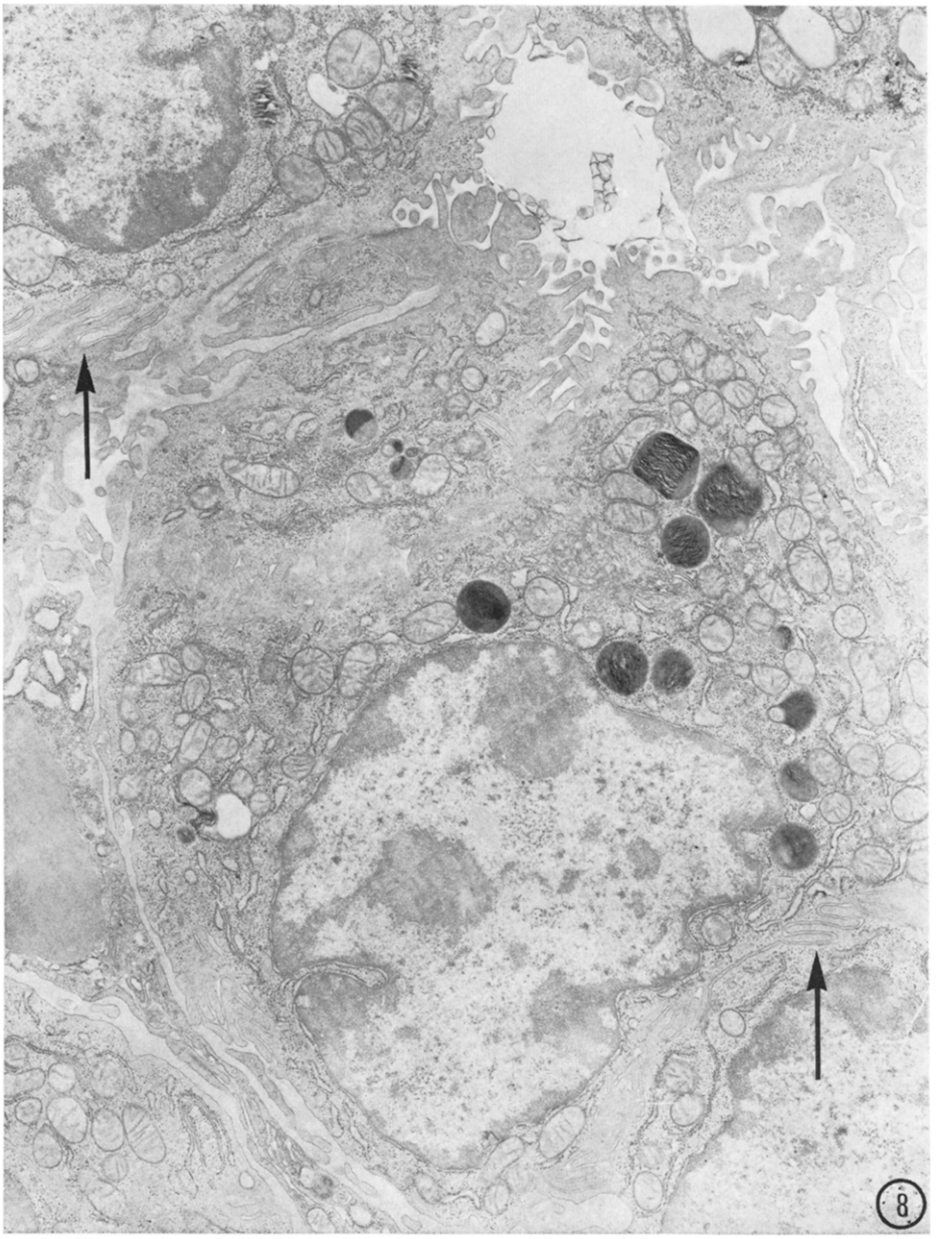


*Fig.* 8. *Intermediate types of adenocarinoma cells. The cytoplasm contains abundant free ribosomes and the cell surface is irregular, showing apical protrusions and lateral interdigitating processes (arrows). Characteristic lamellar inclusion bodies are present, however.* ×13,500.

endoplasmic reticulum were present in all tumour cells, distributed throughout the cytoplasm. This organelle was almost invariably present as single elements, parallel arrays rarely being observed.

The Golgi zones were small but well-developed, consisting of stacks of parallel saccules and associated vesicles. Their distribution within the cytoplasm was apparently random, but there appeared to be some tendency for them to be situated near the nuclear surface.

The tumour-cell cytoplasm contained multivesicular bodies and also a few small dense bodies lacking a lamellar substructure and resembling lysosomal residual bodies or, perhaps, secretion granules.

The mitochondrial population within the cells of these adenocarcinomata exhibited variations in numbers and individual size and form. In some cells there were abundant mitochondria, usually round or oval in profile and of varying diameters. Other cells, however, possessed relatively few mitochondria and in some cases the cristae mitochondriales were reduced in size and number. Few dense intramitochondrial granules were observed. Some mitochondria contained small circular membrane-bounded vesicles (Fig. 3). Mitochondrial forms were also observed which appeared to be in the process of undergoing division (Fig. 3 inset).

The most striking morphological variations observed were those in the structure of the tumour-cell surfaces. The most common surface conformation consisted of relatively smooth basal and lateral aspects, with numerous short microvilli on the apical or acinar surfaces. Minor irregularities of the basal surface were observed, projecting into the loose basement membrane-like material which was present under the tumour epithelium. Bundles of collagen fibres were often observed beneath the basement material. Substantial quantities of subepithelial connective tissue were often present, containing fibroblasts which were normal in appearance. The thickness of this layer varied between wide limits. Tight junctions or terminal bars were present between cells at the sites of contact between their lateral surfaces. However, in some tumour cells the apical cell surface exhibited the presence of irregular cytoplasmic projections protruding into the acinar lumen (Fig. 4). These projections were sometimes quite large (Fig. 5) and contained no organelles, with the occasional exception of a few ribosomes. In some cases the lateral cell surfaces possessed long microvillous processes, closely apposed to, and interdigitating with, those of the neighbouring cells (Fig. 6). Cells possessing either or both types of surface projection usually contained few of the characteristic lamellar inclusion bodies and possessed abundant free ribosomes, in most cases as single unaggregated particles (Fig. 7). Their mitochondria frequently exhibited loss or shortening of the cristae.

Although cells of the type shown in Fig. 7 were morphologically quite dissimilar to those which were more highly differentiated, the common origin of both types was clearly revealed by the existence of intermediate forms (Fig. 8).

Occasionally, tumour cells were observed which appeared to be undergoing degenerative changes. Their granular endoplasmic reticulum was disorganized and they contained autophagic vacuoles.

The nuclei of the tumour cells studied here were round to oval in profile, with minor irregularities of their surfaces. Deep nuclear indentations were sometimes present. Relatively few nuclear pores were observed and the nucleoli were not often prominent. Nuclear inclusions were occasionally observed, morphologically identical to those which were previously described in the similarly induced pulmonary adenoma cells [1].

## DISCUSSION

The general ultrastructural morphology of the pulmonary adenocarcinoma cells described here was similar, in some respects, to that of the previously described adenoma cells [1], which were also induced *in vitro* and which were considered to arise from type II pulmonary alveolar cells [13–16]. The most specific single point of resemblance was the possession of the characteristic lamellar inclusion bodies [17–22] by all three types of cells. A number of previous ultrastructural studies of murine pulmonary tumours arising *in vivo*, have revealed a broadly similar cellular morphology and identical cytogenesis [2–5, 23, 24]. The process of carcinogenesis in mouse lung tissue therefore appears to be accompanied by morphologically similar changes *in vivo* and *in vitro*.

Although considerable variations in cell morphology were observed during the present studies, there was no evidence that more than one tumour cell species was present. All the cells described formed a spectrum showing varying degrees of fine structural deviation from the cell of origin. No evidence was obtained to suggest that any other elements of

the lung tissue, such as the bronchiolar epithelium, might have been involved in tumour formation. This corresponds to the findings of other investigators, previously cited.

Many of the adenocarcinoma cells observed during the present study were almost indistinguishable from the pulmonary adenoma cells which were the subject of a previous report. They contained similar rounded and indented nuclei, with similar, although rather fewer, nuclear inclusions. They possessed a similar mitochondrial population, Golgi apparatus and granular endoplasmic reticulum and frequent cytoplasmic lamellar inclusion bodies of identical morphology. Their ribosomes tended, in general, to occur as polysomal aggregates. The apical cells surfaces usually possessed small microvilli of regular size and form while the lateral surfaces were generally smooth and showed the presence of terminal bars. These cells appeared to represent the more highly differentiated members of the cell population of the adenocarcinomata, especially with respect to their retention of the specific secretory function of the cells of origin. Large quantities of the lamellar inclusion material appeared to be secreted into the "acinar" spaces and accumulated in the lumina of the small cystic structures.

However, the cysts which were observed in the adenocarcinomata were not found in adenomata and their occurrence appears to be one aspect of the relatively greater disorganization of the former tissues. Although it was clear from electron microscopic observations that there was no loss of cell polarity, this was not readily apparent in preparations for optical microscopy. The generally greater irregularity and variation of tissue architecture in the adenocarcinomata was accompanied by the presence of relatively greater quantities of subepithelial connective tissue elements.

The degenerative changes observed in some adenocarcinoma cells were rarely found in adenomata and may reflect a higher rate of cell turnover in the carcinomatous tissues examined here.

The variations in abundance and morphology of the mitochondria in different adenocarcinoma cells did not, in general, appear to be closely related to other ultrastructural variations. However, it is noteworthy that those adenocarcinoma cells which showed the greatest differences from adenoma cells most frequently possessed mitochondria with few or short cristae. The presence of bizarre and "division" forms of mitochondria in some cells indicates a disturbance in the mechanisms of mitochondrial biogenesis.

The state of aggregation and abundance of free cytoplasmic ribosomes did appear to be related, however, to the abundance of specific inclusion bodies and to the presence of surface membrane abnormalities. In chemically induced hepatomata the presence of abundant, unaggregated free ribosomes has been shown to be associated with increased anaplasia [25]. Since free ribosomes are considered to be involved mainly with the synthesis of intracellular or structural proteins, loss of aggregation may reflect either a loss of structural-protein synthetic capacity or, more probably, a change in the type of structural protein being synthesized. The latter view is not inconsistent with the parallel occurrence of changes in the morphology of the surface membrane and dispersal of ribosomes observed during the present study.

Both ribosomal dispersion and changes in the morphology of the tumour-cell surface appeared to be accompanied by a decline in the abundance of cytoplasmic lamellar inclusions. These inclusions are considered to be the unique specific secretion products of type II pulmonary alveolar cells [13–16, 26]. Their presence in pulmonary tumour cells may be regarded as evidence supporting the view that the tumours arise from type II alveolar cells [1, 2, 4, 5] and their relative abundance may reflect the degree of loss of differentiation sustained by such tumour cells. In the present study, therefore, those tumour cells which possessed few lamellar inclusion bodies appear to represent the more anaplastic portion of the tumour-cell population.

The surface changes of the adenocarcinoma cells described here are probably also indicative of increasing malignancy, since they were not observed in the similarly induced non-invasive adenomata [1]. They may be responsible in part for the increased irregularity of the tumour tissue observed by optical microscopy.

Increased irregularity of the cell surface and changes in the morphology of the areas of contact between cells have been shown to accompany carcinogenesis and tumour progression in the liver of the rat [25, 27], in human lung cancer [28], and in cultured cells [29]. The role of changes in the plasma membrane in carcinogenesis was reviewed by Schmidt [30] and, more recently, by Emmelot and Benedetti [31].

The possession of irregular protrusions of the cell surface was, in some cases, the main morphological feature which distinguished the more highly differentiated adenocarcinoma

cells from the cells of the previously described adenomata [1].

It would appear from the present study that the development of an invasive character by murine pulmonary adenomata is accompanied by three main ultrastructural changes, loss of specific inclusion bodies, disaggregation of free ribosomes and increased irregularity of the plasma membrane. There was no evidence to suggest that any one of these changes might arise before the others, although this possibility cannot be exluded entirely. The result of the changes appears to be the formation of tumour cells having a relatively "simplified" ultrastructural organization. It should be noted, however, that even the least well-differentiated tumour cells possessed appreciable quantities of granular endoplasmic reticulum, Golgi zones, a moderate abundance of mitochondria and had retained their polarity. They cannot therefore be regarded as being totally anaplastic. The lesions used for this study were small and could be expected to show the earliest stages of tumour progression. The presence of many cells which were essentially similar to those of non-invasive adenomata suggests that the changes discussed above are those which characterize the development of malignancy in these tumours.

It is evident from these investigations, not only that the initiation of murine pulmonary tumours, similar to those arising spontaneously or induced *in vivo*, may be carried out *in vitro*, but also that the tumours thus produced are capable of becoming malignant. The acquisition of malignancy appears to be accompanied by relatively well-defined ultrastructural changes.

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## SUMMARY

*Mouse lung tissue was treated with* 20-*methylcholanthrene* in vitro *and then implanted into host animals to await the appearance of tumours. The fine structure of pulmonary adenocarcinomata induced by this method was studied and found to resemble, in some respects, that of similarly induced adenomata and of mouse lung tumours arising spontaneously or induced* in vivo.

*Ultrastructural differences between the adenocarcinomata and the previously described adenomata included increased irregularity of cell-surface membranes, disaggregation of ribosomes and decreased numbers of specific cytoplasmic lamellar inclusion bodies. These differences, as well as the greater variations in cell structure which were observed, appeared to be related to the acquisition of a malignant character by these tumours.*

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# Acetylenic Carbamates as Potential Antitumour Agents

E. M. BESSELL, J. A. STOCK and J. H. WESTWOOD

*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W.3.*

## INTRODUCTION

A SERIES of acetylenic carbamates [1] showing experimental antitumour activity has been described and the main structural requirements defined [1, 2]. Significant inhibitory effects were seen only where both $R_1$ and $R_2$ were aryl groups, but substituents $R_3$ and $R_4$ could be varied widely without loss of activity. Derivatives of type (I) also displayed experimental antiviral activity [3, 4]. In a limited trial in human patients, compound (Ia) was effective in a case of giant follicular lymphoma, although other kinds of malignant disease did not respond [5].

In an extension of the American series, we have made a number of structural variants. The amino acid derivatives (Ib–d) were prepared from the corresponding *N*-carbonyl-DL-amino acid methyl esters (II a–c) and the acetylenic alcohol (IIIa), following the published method [1]. The diethoxyphosphinyl derivative (Ie) was made in a similar way. These reactions were carried out at room temperature in the presence of triethylamine (Ic, d, e), or with heating in the presence of sodium acetate (Ib). When *N*-carbonyl-DL-alanine methyl ester was heated with the alcohol (IIIa) and triethylamine, the oxazolidinone (IV) was the major product. That cyclization occurred under these conditions is not surprising [6], but structure (IV) is preferred to the alternative possibility (V) because the n.m.r. spectrum indicates the presence of two exocyclic methylene protons ($\tau$, 5·5, 5·8; $J$, 4Hz; cf. n.m.r. data for the methylene protons of 5,5-diphenyl-3-methyl-4-methyleneoxazolidinone [7]. Our structural assignment also supports an earlier view [8] regarding this type of ring closure.

Attempts to prepare carbamates carrying sugar residues by reaction of alcohol (IIIa) with appropriate protected isocyanates were unsuccessful [9].

The carbamates described thus far are o structure (I). We have in addition prepared two types of dicarbamates (VI and VII) by reaction of ethyl isocyanate with the corresponding diols (VIII and IX). The fluorinated derivatives were made because of the high potency of the *p*-fluoro compounds in the original series [1].

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## CHEMICAL SYNTHESIS

Melting points were determined on a Köfler block and are corrected; n.m.r. spectra were recorded on a 60 MHz Perkin–Elmer R-10 spectrometer; $CDCl_3$ was used as solvent and tetramethylsilane as internal standard. The chemical shifts are given in ppm on the $\tau$ scale and coupling constants ($J$) in Hz (Hertz). Gas–liquid chromatography (g.l.c.) was carried out on a Pye chromatograph (series 104, model 4), and Merck Kieselgel G was used in the thin-layer chromatography (t.l.c.).

1,1-*Diarylprop*-2-*yn*-1-*ols* (III)

The method of Hartzler [10] was used, starting from the appropriate diaryl ketone. It was advisable not to expose the liquid ammonia to the air for longer than was absolutely necessary, otherwise conversion was incomplete and the alcohol, contaminated with starting ketone (as shown by g.l.c.), was diffi-

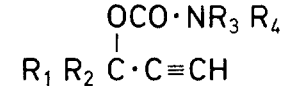
cult to crystallize. In such cases, the alcohol was obtained pure by repeating the synthetic procedure with the impure product.

*N-Carbonyl-DL-amino acid methyl esters* (II)

High yields (ca.90%) were obtained, following the method of Goldschmidt and Wick [11]. The alanine derivative (IIa) had b.p. 54°/5 mm; Schlögl [12] reported b.p. 82–84°/18 mm. The structures of the valine and methionine *derivatives* (IIb, c; b.p. 79°/10 mm and 114°/0·8 mm respectively) were confirmed by n.m.r. (IIb): OCNCH, 6·0 (doublet; $J$ 4·3); $OCH_3$, 6·2 (singlet); CH (in $Pr^i$), 7·7 (multiplet); $CH_3$ (in $Pr^i$) 8·95 (doublet; $J$ 7); 2nd $CH_3$ (in $Pr^i$), 9·05 (doublet; $J$ 7). (IIc): CH, 5·7 (multiplet); $OCH_3$ 6·2 (singlet); $SCH_2$ 7·4 (multiplet); $SCH_3$, 7·9 (singlet); $CH_2$ 7·9 (multiplet).

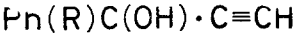
*Carbamates carrying amino acid-ester residues (Ib–d)*

The valine and methionine derivatives (Ic,d) were prepared by a procedure similar to method C of Dillard *et al.* [1]. For example, a mixture of 1,1-diphenylprop-2-yn-1-ol (IIIa; 0·6 g), *N*-carbonylmethionine methyl ester (3 g), triethylamine (1 ml), and alcohol-free chloroform (10 ml) was set aside at room temperature. The reaction was monitored by t.l.c. (ether–petrol, 1:1). After three days, no starting material was detectable. The residue from evaporation of the reaction mixture was chromatographed on Merck Kieselgel (0·05–0·2 mm particles), with ether–petrol (2:3) as eluant. Two successive crystallizations of the major product from benzene-cyclohexane gave the required *carbamate* (Id, 0·35 g; 30%), m.p. 89–90° (Found: C, 66·7; H, 5·9; N, 3·6; S, 8·2. $C_{22}H_{23}NO_4S$ requires C, 66·5; H, 5·8; N, 3·5; S, 8·1%), n.m.r.: $Ph_2C$, 2·7 (multiplet); NH, 4·4 (doublet; $J$ 8); NCH 5·6 (multiplet); $OCH_3$ 6·3 (singlet); C≡CH, 7·0 (singlet); $SCH_2$, 7·6 (multiplet); $SCH_3$, 8·0 (singlet); $CH_2$, 8·0 (multiplet). The *valine derivative* (Ic), made in a similar way, had m.p. 146–7° (benzene-cyclohexane; 75% yield) (Found: C, 72·2; H, 6·3; N, 3·8. $C_{22}H_{23}NO_4$ requires: C, 72·4; H, 6·3; N, 3·8%), n.m.r.: $Ph_2C$, 2·7 (multiplet); NH, 4·5 (doublet; $J$ 10); NCH, 5·7 (quartet; $J$ 10, 5); $OCH_3$, 6·3 (singlet); C=CH, 7·0 (singlet); CH, 7·9 (multiplet); $CH_3$ (in $Pr^i$), 9·1 (doublet; $J$ 7); 2nd $CH_3$ (in $Pr^i$), 9·15 (doublet; $J$ 7). The *alanine derivative* (Ib) was prepared in the same way as the *N*-ethyl derivative (Ij; see below). It had m.p. 130–131° (benzene-cyclohexane; 40% yield). (Found: C, 71·0; H, 5·6; N, 3·8. $C_{20}H_{19}NO_4$ requires C, 71·0; H, 6·0; N, 4·1%); n.m.r.: $Ph_2C$, 2·6 (multiplet); NH, 4·4 (doublet; $J$ 7); CH, 5·6 (quintet);

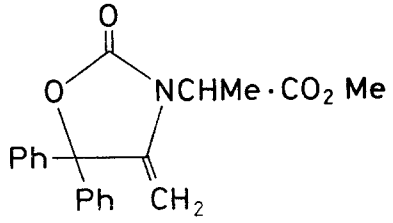


FORMULA (I). (a) – (j) $R_1$=Ph; (a) – (h) $R_2$=Ph; (a) $R_3$=H, $R_4$=cyclohexyl; (b) $R_3$=H, $R_4$=CHMe$CO_2$Me; (c) $R_3$=H, $R_4$=CH$Pr^i$$CO_2$Me; (d) $R_3$=H, $R_4$=CH($CH_2CH_2$SMe)$CO_2$Me; (e) $R_3$=H, $R_4$=$(EtO)_2PO$; (f) $R_3$=$R_4$=H; (g) $R_3$+$R_4$=$(CH_2)_5$; (h) $R_3$=H, $R_4$=Et; (i) $R_2$=*p*$FC_6H_4$, $R_3$=H, $R_4$=cyclohexyl; (j) $R_2$=*p*$ClC_6H_4$, $R_3$=H, $R_4$=Et.

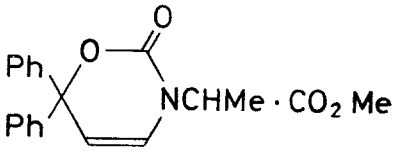
FORMULA (II). (a) R=Me; (b) R=$Pr^i$; (c) R=$CH_2CH_2$SMe.



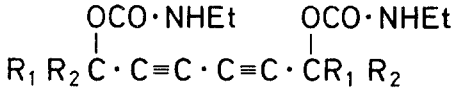
FORMULA (III). (a) R=Ph; (b) R=*p*$ClC_6H_4$ (c) R=*p*$FC_6H_4$.



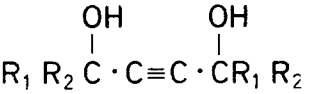
FORMULA (IV).



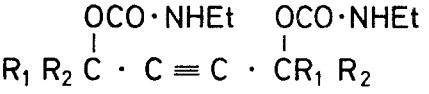
FORMULA (V).



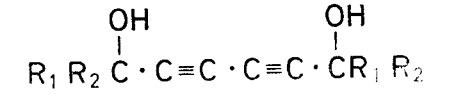
FORMULA (VI). (a) $R_1$=R=$_2$Ph; (b) $R_1$=Ph, $R_2$=*p*$FC_6H_4$.



FORMULA (VII). (a) $R_1$=$R_2$=Ph; (b) $R_1$=Ph, $R_2$=*p*$FC_6H_4$.



FORMULA (VIII). (a) $R_1$=$R_2$=Ph; (b) $R_1$=Ph, $R_2$=*p*$FC_6H_4$.



FORMULA (IX). (a) $R_1$=$R_2$=Ph; (b) $R_1$=Ph, $R_2$=*p*$FC_6H_4$.

*Table* 1. *Dicarbamates*

| Compound | M.p. (°C) | Yield (%) | Formula | Found C | Found H | Found F | Found N | Requires C | Requires H | Requires F | Requires N |
|---|---|---|---|---|---|---|---|---|---|---|---|
| VIa | 152–154* | 60 | $C_{34}H_{32}N_2O_4$ | 76·4 | 6·1 | — | 5·2 | 76·7 | 6·0 | — | 5·3 |
| VIb | 127–128* | 65 | $C_{34}H_{30}F_2N_2O_4$ | 72·2 | 4·9 | 6·3 | 5·0 | 71·9 | 5·3 | 6·7 | 4·9 |
| VIIa | 232–233† | 90 | $C_{36}H_{32}N_2O_4$ | 77·2 | 5·7 | — | 4·9 | 77·7 | 5·8 | — | 5·0 |
| VIIb | 219–220* | 60 | $C_{36}H_{30}F_2N_2O_4$ | 72·9 | 5·0 | 6·7 | 4·7 | 73·0 | 5·1 | 6·4 | 4·7 |

*From benzene.
†From ethyl acetate.

$OCH_3$, 6·3 (singlet); C≡CH, 7·0 (singlet); $CH_3$, 8·6 (doublet; $J$ 7).

N-(*Diethoxyphosphinyl*)-1,1-*diphenylprop*-2-*ynyl carbamate* (*Ic*)

This carbamate was prepared from diethylphosphinyl isocyanate [13] by the method used for the preparation of the valine and methionine compounds (see above). The *product* (60%) had m.p. 129–130° (benzene–petrol). (Found: C, 61·8; H, 5·8; N, 3·7; P, 8·2 $C_{20}H_{22}NO_5P$ requires C, 62·0; H, 5·7; N, 3·6; P, 8·0%); n.m.r.: $Ph_2C$, 2·7 (multiplet); NH, 2·2 (doublet); $OCH_2$, 5·9 (quintet); C≡CH, 7·0 (singlet); $CH_3$, 8·75 (triplet).

N-*Ethyl*-1-(4-*chlorophenyl*)-1-*phenylprop*-2-*ynyl carbamate* (Ij)

The procedure was essentially that of Shapiro *et al.* [6]. A mixture of the corresponding alcohol (IIIb; 4·0 g), ethyl isocyanate (1·5 g) and anhydrous sodium acetate (0·1 g) was heated on a steam bath for 5 hr. Ether (30 ml) was added to the cooled mixture. Evaporation of the filtrate gave an oil which, on two successive crystallizations from ethyl acetate–petrol, gave the *carbamate* (1·9 g; 38%), m.p. 116° (Found: C, 68·8; H, 5·1; Cl, 11·2; N, 4·3. $C_{18}H_{16}ClNO_2$ requires: C, 69·0; H, 5·1; Cl, 11·3; N, 4·5%); n.m.r.: $Ph_2C$, 2·7 (multiplet); NH, 5·0 (triplet); $CH_2$, 6·8 (quintet); C≡CH, 7·0 (singlet); $CH_3$, 8·9 (triplet).

5,5-*Diphenyl*-3-(1-*methoxycarbonylethyl*)-4-*methyleneoxazolidin*-2-*one* (IV)

The method of preparation resembled that of the valine and methionine derivatives (Ic, d), but toluene, not chloroform, was used as solvent. *N*-carbonyl-DL-alanine methyl ester (prepared *in situ*) and diphenylprop-2-ynol were heated under reflux for 5 hr. Evaporation gave an oil which crystallized from ethyl acetate–petrol, giving the *oxazolidinone*, m.p. 96°, in high yield. (Found: C, 70·8; H, 5·65; N, 4·2. $C_{20}H_{19}NO_4$ requires: C, 71·0; H, 5·95; N, 4·1%); n.m.r.: $Ph_2C$, 2·6 (multiplet); NCH, 5·2 (quartet); $C{=}CH_2$, 5·5, 5·8 (quartet; $J$ 6); $OCH_3$, 6·3 (singlet); C–$CH_3$, 8·4 (doublet; $J$ 8).

1,1,4,4-*Tetra-arylbut*-2-*yne*-1,4-*diols* (VIII)

The method of Beumel and Harris [14] was used. The fluorinated *diol* (VIIIb), m.p. 160–161° (benzene–petrol), was thus prepared from *p*-fluorobenzophenone in 65% yield. (Found: C, 78·6; H, 5·0; F, 9·0. $C_{28}H_{20}F_2O_2$ requires: C, 78·9; H, 4·7; F, 8·9%).

1,1,6,6-*Tetra-arylhexa*-2,4-*diyne*-1,6-*diols* (IX)

The procedure of Chodkiewicz [15] was used. The fluorinated *diol* (IXb), m.p. 96–7° (benzene–petrol), was prepared in 70% yield from the acetylenic alcohol (IIIc). (Found: C, 80·1; H, 4·7; F, 8·7. $C_{30}H_{20}F_2O_2$ requires: C, 80·0; H, 4·4; F, 8·4%).

*Dicarbamates* (VI, VII)

The reaction between the appropriate diol and ethyl isocyanate in the presence of triethylamine was carried out at room temperature as in the preparation of the amino acid derivatives (Ic, d). Data are given in Table 1.

## PHARMACOLOGICAL METHODS

The plasma cell tumour assay method resembled that described previously [16]. Balb/C- mice (male or female, but not mixed in any one test; tumour growth rate tended to be greater in the females) were implanted with the ADJ/PC6A plasma cell tumour subcutaneously by trocar. The animals were not treated until the tumours were well established and readily palpable (17–28 days after transplantation; tumour mass ca.1 g). Compounds were injected intraperitoneally as single doses in arachis oil or arachis oil–acetone (9: 1). Groups of three mice were used for each dose level and the untreated tumour control groups each comprised 11 animals. Ten or 11 days

*Table 2. Antitumour potency and toxicity of a series of acetylenic carbamates and of an oxazolidinone*

| Compound | Dose (mg/kg) | PC6 tumour inhibition (%)* | Mortality | LD50 (mg/kg) | ID90 (mg/kg) | Therapeutic index |
|---|---|---|---|---|---|---|
| Ia† | 3·3 | 13 | 0/3 | 68 | 23 | 3 |
| | 10 | 60 | 0/3 | | | |
| | 30 | 100 | 0/3 | | | |
| | 90 | 100 | 2/3 | | | |
| | 180 | — | 3/3 | | | |
| Ib | 3·3 | −7 | 0/3 | >90 | — | — |
| | 10 | 12 | 0/3 | | | |
| | 30 | 89 | 0/3 | | | |
| | 90 | 58 | 1/3 | | | |
| Ic | 10 | −16 | 0/3 | >320 | 140 | >2 |
| | 20 | −50 | 0/3 | | | |
| | 40 | 9 | 0/3 | | | |
| | 80 | 70 | 0/3 | | | |
| | 160 | 100 | 0/3 | | | |
| | 320 | 100 | 1/3 | | | |
| Id | 10 | −14 | 0/3 | 113 | — | — |
| | 20 | −39 | 0/3 | | | |
| | 40 | −4 | 0/3 | | | |
| | 80 | 27 | 0/3 | | | |
| | 160 | — | 3/3 | | | |
| Ie | 5 | 15 | 0/3 | 23 | — | — |
| | 10 | −4 | 0/3 | | | |
| | 20 | −6 | 1/3 | | | |
| | 40 | — | 3/3 | | | |
| If† | 6·6 | 48 | 0/3 | 52 | 26 | 2 |
| | 10 | 8 | 0/3 | | | |
| | 30 | 100 | 0/3 | | | |
| | 90 | | 3/3 | | | |
| Ig† | 6·6 | −24 | 0/3 | 128 | 68 | 2 |
| | 10 | 43 | 0/3 | | | |
| | 30 | 62 | 0/3 | | | |
| | 90 | 100 | 0/3 | | | |
| | 180 | — | 3/3 | | | |
| Ih† | 10 | −20 | 0/3 | 135 | 25 | 5 |
| | 20 | 25 | 0/3 | | | |
| | 40 | 100 | 0/3 | | | |
| | 80 | 100 | 0/3 | | | |
| | 160 | 100 | 2/3 | | | |
| | 320 | — | 3/3 | | | |
| Ii† | 22 | 67 | 0/3 | 38 | — | — |
| | 66 | — | 3/3 | | | |
| Ij | 10 | 0 | 0/3 | 170 | 45 | 4 |
| | 30 | 60 | 0/3 | | | |
| | 60–120 | 100 | 0/3 | | | |
| | 240 | — | 3/3 | | | |
| IV | 81 | −55 | 0/3 | >243 | — | — |
| | 243 | 7 | 0/3 | | | |
| VIa | 80 | 27 | 0/3 | >1280 | — | — |
| | 160 | 34 | 0/3 | | | |
| | 320 | 25 | 0/3 | | | |
| | 640 | —‡ | 0/3 | | | |
| | 1280 | —‡ | 0/3 | | | |

*Table 2 continued*

| Compound | Dose (mg/kg) | PC6 tumour inhibition (%)* | Mortality | $LD_{50}$ (mg/kg) | $ID_{90}$ (mg/kg) | Therapeutic index |
|---|---|---|---|---|---|---|
| VIb | 73 | −16 | 0/3 | | | |
| | 145 | −17 | 0/3 | | | |
| | 290 | −32 | 0/3 | >1160 | — | — |
| | 580 | −13 | 0/3 | | | |
| | 1160 | −22 | 0/3 | | | |
| VIIa | 100 | −2 | 0/3 | | | |
| | 300 | 40 | 0/3 | >900 | — | — |
| | 900 | −38 | 0/3 | | | |
| VIIb | 100 | −11 | 0/3 | | | |
| | 300 | −32 | 0/3 | >900 | — | — |
| | 900 | 13 | 0/3 | | | |

*Values of less than 40 are not considered significant. Negative values indicate that the mean tumour weight exceeded that of the controls.
†Dillard *et al.* [1].
‡Visual examination of tumours only; no apparent inhibition.

after drug treatment, the mice were killed, and the tumours dissected out and weighed. The $LD_{50}$ and $ID_{90}$ (dose giving 90% tumour inhibition) were determined graphically; the therapeutic index is defined in this work as $LD_{50}/ID_{90}$.

The L1210-mouse leukaemia screening test followed the standard procedure at this Institute [17]. Briefly, C57/DBA2 F1 hybrids were inoculated subcutaneously with standard titres of leukaemic spleen cells. Five daily doses of the compounds in arachis oil were given intraperitoneally, treatment beginning 24 hr after tumour inoculation. Groups of 5 mice were used for each dose level, and methotrexate-treated controls were included to check the sensitivity of the tumour. The three dose levels used for each of the three carbamates tested (Ia, f, g) were based on preliminary chronic-toxicity determinations (10 daily doses). Drug activity was assessed by the increase, if any, in average survival time over that of the control mice, which die on average 11 days after tumour inoculation under the conditions used in these laboratories.

## RESULTS AND DISCUSSION

Dillard and co-workers [1, 2] used leukaemia C1498 and the plasma cell tumour X5563 in mice, with a multiple dose regimen, to evaluate their carbamates. Neither of these tumours is maintained at this Institute. It was found, however, that the established ADJ/PC6A plasma cell tumour in mice responded to single doses of each of 5 representative compounds (Ia, f–i) reported by the American group as tumour-inhibitory. On the other hand, multiple daily doses of compounds (Ia), (If) and (Ig), each tested at three dose levels up to the toxic range, failed to increase the average survival time of mice previously inoculated with L1210 leukaemia; the chronic $LD_{50}$ values in random-bred female mice for carbamates (Ia), (If) and (Ig) were 56, 35 and 56 mg/kg/day × 10 respectively. The PC6 tumour system was therefore used to compare the activities of the compounds described here. The detailed results are set out in Table 2.

Four compounds (Ia, f–h) from the earlier series [1] caused complete tumour regression at tolerated doses. The fifth carbamate (Ii) of this group was not fully assessed, but it is clear that the *p*-fluoro group imparts no therapeutic advantage in the PC6 test system. In the same way, the *N*-ethyl carbamate (Ih) and its *p*-chloro analogue (Ij) were approximately equal in potency. These two compounds showed the (for this series) relatively high therapeutic indices of 5 and 4 respectively.

Of the amino acid derivatives, only the valine compound (Ic) caused complete regression at a non-lethal dose, but the therapeutic index was unremarkable. The alanine derivative (Ib) gave a degree of inhibition, but neither the methionine analogue (Id) nor the phosphinyl derivative (Ie) affected the PC6 tumour at tolerated doses.

Compounds (IV), (VI) and (VII) were apparently devoid of activity, but toxic dose levels were not established. The lack of activity of the oxazolidinone (IV) is not surprising, because cyclized compounds exa-

mined by Shapiro and others [6] and now believed (see Introduction) to be oxazolidinones analogous to compound (IV), showed no anticonvulsant activity; in this they differed from the corresponding open chain acetylenic carbamates (I:$R_1$ and $R_2$=alkyl).

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## SUMMARY

*A number of acetylenic mono- and dicarbamates has been prepared. Activity against the ADJ/PC6A plasma cell mouse tumour was seen only among the monocarbamates. Three of the active derivatives were separately tested against the L1210 leukaemia in mice but failed to increase the average survival time. A related oxazolidinone did not inhibit the plasma cell tumour.*

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# Chromosomal Patterns in Human Meningiomas

JOACHIM MARK

*Institute of Pathology, Department of Neurosurgery and Institute of Genetics, University of Lund, Lund, Sweden*

## INTRODUCTION

DURING the last 10 years the knowledge of chromosomal conditions in human malignant tumours has rapidly increased. However, still very little is known about the cytogenetical features of benign neoplasms in the human. Observations in such neoplasms were reviewed in a recent report which dealt with the chromosomal findings in two benign, intracranial human tumours, one chromophobic pituitary adenoma and one angioblastic meningioma [1]. The present investigation is an extension of this previous study. Karyological findings in 12 additional benign human tumours, all of them meningiomas, are presented below. The results will be considered together with earlier chromosomal observations in meningiomas [1–3] in order to characterize the stemline pattern, to evaluate the specificity of numerical and structural aberrations and for a comparison with the chromosomal deviations in a group of primary malignant human neoplasms [4].

## MATERIAL AND METHODS

Table 1 shows the clinical and pathological characteristics of the material. All of the 12 meningomas, except M2, occurred in adults, and M2 was also the only tumour with a spinal location. None of the patients was treated with X-rays or cytostatics before the operation. Usually the whole tumour could be excised. Histopathologically four types of meningiomas were distinguished [5], syncytial, transitional, fibroblastic and angioblastic types.

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All meningiomas were studied both in fixations directly from the tumour (D) and in fixations from material explanted *in vitro* (Pc). In three cases, M2, M7 and M12, early *in vitro* passages (P) were used for further fixations. The methods for D and Pc were described previously [1, 6].

Twenty metaphases or more were counted in all fixations. Karyotype analyses were made by photography. The number of karyotyped cells was 15–40 in all tumours. Markers were classified and denoted with symbols in accordance with the nomenclature proposed by Levan, *et al.* [7]. The terms stemline and sideline were defined earlier [1, 4, 6]; S and s ($s_1$, $s_2$, etc), respectively, were used as symbols for them.

## RESULTS

Table 2 shows the chromosome counts in all fixations. A successful fixation directly from the tumour (D) was obtained only in two cases, M1 and M12; in both instances the results agreed with the findings in the fixation from the primary culture, Pc-6 and Pc-3, respectively. In the other 10 cases, M2–M11, the analyses were based on findings in fixations from primary cultures after a duration of 3–6 days.

The karyotypic features of the stem-sidelines in the meningomas are illustrated in Table 3 (findings in *in vitro* passages excluded). The last column in the table shows the estimated frequency of stem line and sideline cells in the tumour cell population. The frequencies could easily be calculated from the results of the karyotype analyses which were random samples.

*M1*. This remarkable hypodiploid menin-

*Table* 1. *Clinico-pathological features of the material*

| Meningioma No. | Age of the patient, years | Sex of the patient | Location of the meningioma | Histological type of the meningioma |
|---|---|---|---|---|
| M 1 | 68 | F | Right tempero-parietal region | Fibroblastic |
| M 2 | 14 | F | Spinal canal, $C_6$-$Th_1$ | Transitional |
| M 3 | 60 | F | Right cerebello-pontine angle | Transitional |
| M 4 | 67 | M | Right fossa Sylvii | Syncytial |
| M 5 | 62 | F | Left parieto-frontal region | Fibroblastic |
| M 6 | 63 | M | Roof of the 4th ventricle | Transitional |
| M 7 | 68 | F | Left parietal region | Transitional |
| M 8 | 42 | F | Left fossa Sylvii | Angioblastic |
| M 9 | 64 | F | Right parieto-frontal region | Syncytial |
| M10 | 56 | M | Left subfrontal region | Transitional |
| M11 | 61 | M | Left parietal region | Syncytial |
| M12 | 47 | M | Left parietal region | Transitional |

goma, with S=41, had as many as 5 markers in the stem line (Fig. 1a). The marker set consisted of three metacentrics, $M_1$, $M_2$ and $M_3$, 1 submetacentric, sm, and 1 acrocentric, t. The size of $M_1$ was approximately half of that of the chromosomes Nos. 19–20; the arms of $M_1$ seemed to be of equal size though measurements were very difficult due to the small size of the marker. $M_2$ and $M_3$ had a similar size; they were a little smaller than the chromosomes No. 16 but could be distinguished from them due to the median location of the centromere. For unknown reasons one or sometimes both of these markers showed a distended centromeric region (Fig. 1a). The sm-marker had an arm-index of about 1·7 and it was approximately 25% longer than the chromosomes No. 2. The t-marker was about 25% longer than the biggest members in group D, and the short arms often carried clear satellites.

The morphology of the markers was the same in all S-cells. One or two of the markers were missing in several variant cells but those present had characteristics as in the S-cells. In a few variant cells a sixth marker was seen; it was of the t-type and was a little smaller than the smallest members in group D.

The S-cells had lost three G chromosomes, as had the majority of the variant cells. However, 10 of the 38 karyotyped cells had lost only one G chromosome. Another consistent feature in these 10 cells was the absence of one of the markers $M_2$–$M_3$. The chromosome numbers in this deviating group varied between 39 and 43 but 6 cells had 42 chromosomes. Though this cell population does not fulfil the criteria for a sideline it is biologically comparable to an s.

Four hypomodal cells with chromosome numbers between 32 and 37 were analyzed. As to losses in group G and composition of the marker set they were closely related to the S-cells but no consistent feature could be found in the additional losses. Thus, it is possible that they represent broken metaphases. The polyploid cells, however, had karyotypes in accordance with doubling-products of S-cells and related variant cells.

As in subsequent meningiomas, the frequency of breakage was low (both in D and Pc) only about 5%. This low frequency, as well as the morphological stability of the markers, indicated that the structural rearrangements had occurred a considerable time prior to the present analysis. The details of these rearrangements could not be deduced. However, it was obvious that one of the small or big acrocentrics had participated in the formation of the t-marker, and it seemed reasonable to assume that, especially, group C was involved in the formation of the other markers.

*M2, M3, M4 and M5.* These four meningiomas all had an S-number of 45 and their stemline showed the same deviation, a loss of one G chromosome (Figs. 1b, 1c). M4 was a male tumour but karyotype analyses indicated that the Y chromosome was present. Thus the loss in all meningiomas concerned one member of the pairs Nos. 21–22.

*In M2* all 45-chromosome cells analyzed


*Fig. 1. Karyotypes in meningiomas M1, M3 and M4.*


*Fig. 2. Karyotypes in meningiomas M6, M7, M8 and M9.*

(to face p. 490)

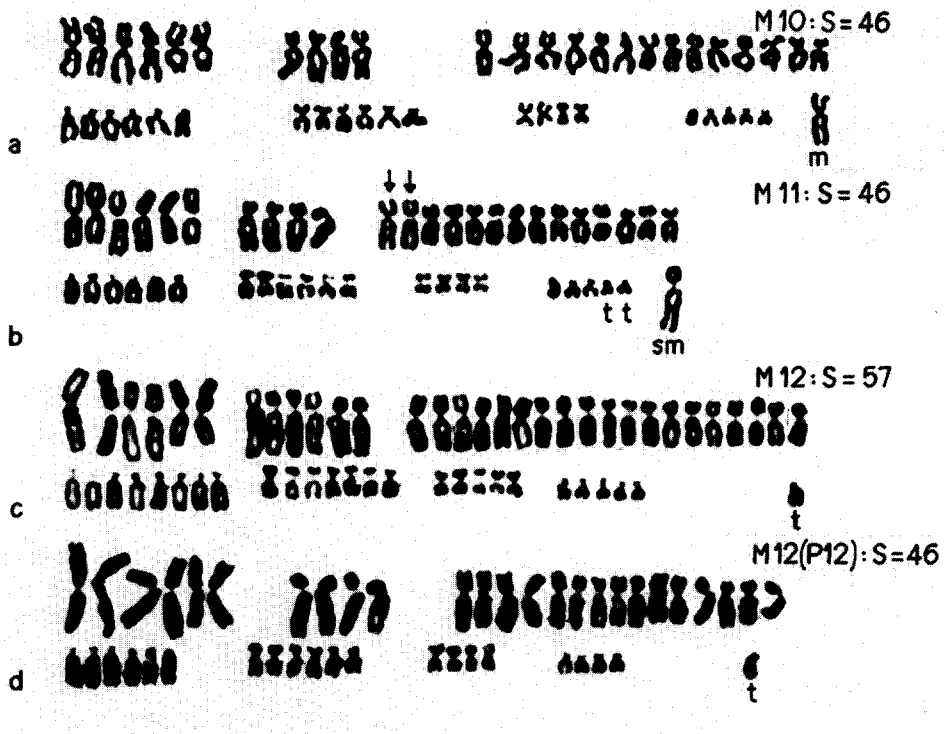


*Fig. 3. Karyotypes in meningiomas M10, M11 and M12.*

*Table 2. Chromosome counts*

| Meningioma (M) No. | *In vitro* Passage (P) No. | D or Pc-days | Chromosome numbers | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | Total counts |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 32–38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 2x 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 73 | 78 | 82 | 88 | 90 | 92 | 94 | 115–184 | | | | | | |
| M 1 | | D | 4 | 3 | 4 | 17 | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | — | — | — | — | — | | | | | | 31 |
| | | Pc- 6 | 5 | 4 | 9 | 40 | 8 | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | 1 | — | — | — | — | — | | | | | | 70 |
| M 2 | | Pc- 3 | — | — | — | — | 1 | 2 | 10 | 47 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | | | | 60 |
| | P 1 | Pc- 18 | — | — | — | — | — | 2 | 3 | 25 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | | | | 30 |
| | P 2 | Pc- 32 | — | — | — | — | — | 1 | 5 | 30 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | | | | 36 |
| | P10 | Pc- 94 | — | — | — | — | — | 2 | 6 | 18 | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | 1 | — | — | — | | | | | | 30 |
| M 3 | | Pc- 3 | — | — | — | — | 1 | — | 1 | 23 | 13 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | | | | 38 |
| M 4 | | Pc- 6 | — | — | — | — | — | — | 4 | 27 | 19 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | | | | 50 |
| M 5 | | Pc- 4 | — | — | — | — | 2 | 1 | 10 | 45 | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | | | | 60 |
| M 6 | | Pc- 3 | — | — | — | — | 1 | — | 2 | 28 | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | — | — | — | — | — | — | — | | | | | | 34 |
| M 7 | | Pc- 4 | — | — | — | — | — | — | — | 40 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | | | | 40 |
| | P 7 | Pc- 74 | — | — | — | 1 | — | 2 | 4 | 32 | 1 | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | 4 | 5 | — | — | — | | | | | | 51 |
| M 8 | | Pc- 4 | — | — | 1 | — | 4 | 1 | 4 | 59 | — | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 2 | — | — | — | | | | | | 72 |
| M 9 | | Pc- 3 | — | — | — | — | 1 | 1 | 7 | 1 | 18 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | | | | 28 |
| M10 | | Pc- 3 | — | — | — | — | — | 1 | 4 | 5 | 38 | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | — | — | | | | | | 50 |
| M11 | | Pc- 6 | — | — | — | — | — | 1 | 1 | 2 | 46 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 3 | — | — | | | | | | 53 |
| M12 | | D | — | — | — | — | — | 1 | — | — | 2 | 1 | 1 | 1 | 1 | — | 1 | 2 | 1 | — | 2 | 15 | 6 | 1 | — | — | — | — | — | — | — | — | — | — | | | | | | 35 |
| | | Pc- 3 | — | — | — | — | — | — | 2 | — | — | — | — | 1 | — | — | 1 | 3 | 1 | — | 10 | 26 | 8 | — | — | — | — | — | — | — | — | — | — | 1 | | | | | | 53 |
| | P 1 | Pc- 11 | — | — | — | — | — | — | — | — | — | 1 | 1 | 2 | — | — | — | 2 | — | 6 | 7 | 8 | 22 | 6 | 2 | — | — | — | — | — | — | — | — | 3 | | | | | | 60 |
| | P 2 | Pc- 16 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | — | — | — | 6 | 14 | 2 | 1 | 1 | — | — | — | — | — | — | — | — | | | | | | 25 |
| | P11 | Pc- 96 | — | — | — | — | — | — | — | — | 32 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | | | | 32 |
| | P12 | Pc-112 | — | — | — | — | — | — | — | 8 | 21 | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | — | — | — | — | — | — | — | — | — | | | | | | 30 |

*Table 3. Survey of chromosomal characteristics of stem-sidelines and their frequency in the tumour cell population*

| Meningioma No. | Stemsideline numbers | Chromosome groups | | | | | | | | | | Markers | Percentage s- and s-cells in the tumour cell population |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | A 2 | 3 | B 4–5 | C 6–X–12 | D 13–15 | 16 | E 17–18 | F 19–20 | G Y–21–22 | | |
| M 1 | S=41 | −1 | – | – | – | -5 | −1 | – | – | – | −3 | +1M$_1$, +1M$_2$, +1M$_3$, +1sm, +1t | 37 |
| | (s=42 | +1 | – | – | – | –5 | −1 | – | – | – | −1 | +1M$_1$, +1M$_2$, +1sm, +1t | 8) |
| M 2 | S=45 | – | – | – | – | – | – | – | – | – | −1 | – | 78 |
| | s=44 | – | – | – | – | −1 | – | – | – | – | −1 | – | 17 |
| M 3 | S=45 | – | – | – | – | – | – | – | – | – | −1 | – | 61 |
| | s=46 | – | – | – | – | – | – | – | – | – | – | – | 34 |
| M 4 | S=45 | – | – | – | – | – | – | – | – | – | −1 | – | 50 |
| | s=46 | – | – | – | – | – | – | – | – | – | – | – | 25 |
| M 5 | S=45 | – | – | – | – | – | – | – | – | – | −1 | – | 75 |
| | s=44 | – | – | – | – | −1 | – | – | – | – | −1 | – | 10 |
| M 6 | S=45 | – | – | – | – | – | −1 | – | – | – | – | – | 82 |
| M 7 | S=45 | – | – | – | – | – | – | – | −1 | – | −1 | +1t | 80 |
| M 8 | S=45 | – | −1 | +1 | −1 | – | −1 | – | −1 | – | −2 | +1m, +1st, +1t$_1$, +1t$_2$ | 63 |
| M 9 | S=46 | – | – | – | – | +1 | −1 | – | – | +1 | −1 | – | 64 |
| | s=44 | – | – | – | – | – | −1 | – | – | – | −1 | – | 25 |
| M10 | S=46 | – | – | – | – | −1 | – | – | – | – | – | +1m | 68 |
| M11 | S=46 | – | – | – | – | −1 | – | – | – | – | −2 | +1sm, +2t | 79 |
| M12 | S=57 | – | – | – | +2 | +4 | +2 | – | +1 | +1 | – | +1t | 37 |
| M13* | S=47 | – | – | – | – | – | – | – | – | – | – | +1t$_1$ | 55 |
| | s=48 | – | – | – | – | – | – | – | – | – | – | +1t$_1$, +1t$_2$ | 15 |

*Reported in detail earlier [1].

*Table 4. Mean differences found–expected*

| Material | Number of tumours | Chromosome types | | | | | | | | | | Markers |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4–5 | 6–X–12 | 13–15 | 16 | 17–18 | 19–20 | Y–21–22 | |
| Present material — No markers | 6 | +0·0 | +0·0 | +0·0 | +0·1 | +0·5 | −0·2 | +0·0 | +0·1 | +0·3 | −0·8 | ±0 |
| Present material — Markers | 7 | −0·2 | −0·2 | +0·1 | +0·1 | −0·6 | −0·1 | −0·0 | −0·2 | +0·1 | −1·2 | +2·3 |
| Present material — Total | 13 | −0·1 | −0·1 | +0·1 | +0·1 | −0·1 | −0·2 | −0·0 | −0·1 | +0·2 | −1·0 | +1·2 |
| Zang and Singer's material [2] | 8 | +0·1 | +0·1 | +0·1 | +0·1 | +0·4 | −0·3 | +0·0 | +0·1 | +0·1 | −0·7 | ±0 |
| All meningiomas | 21 | −0·0 | −0·0 | +0·1 | +0·1 | +0·1 | −0·2 | +0·0 | −0·0 | +0·2 | −0·9 | +0·8 |
| Primary neurogenic tumours in children [4] | 22 | +0·1 | +0·0 | −0·2 | −0·0 | +0·4 | −0·4 | +0·0 | −0·1 | −0·1 | −0·5 | +0·8 |

had the same karyotype. The spread was exclusively hypomodal. Its true character was indicated by the s=44 and the restriction of the losses in the variant cells to groups C, D and G. In the passages of M2, P1, P2 and P10 the stem line was unchanged and the majority of the variant cells analyzed had the same characteristics as in Pc-3. In P10 they again formed an s=44. The two 46-chromosome cells in P10 had, a normal, diploid karyotype. The hypotetraploid cells had karyotypes which agreed with doubling-products of S- and s-cells, respectively. Markers were only seen in one cell in one fixation (P10). This 43-chromosome cell had lost one C, one D and two G chromosomes and gained two t-markers. Both markers were about half as big as the chromosomes Nos. 21–22 and they seemed to be satellited; their morphology indicated that they could represent deleted D or G chromosomes.

*In M*3 all the 45-chromosome cells analyzed had the same karyotype, too, and the two hypomodal variant cells showed additional losses in groups C and D. The 46-chromosome cells studied had all a normal diploid karyotype and they constituted an s. No cell with a marker was seen.

*In M*4 only one of the 13 analyzed cells with the S-number had a deviating karyotype; this cell had lost one chromosome No. 2 and 1 Nos. 17–18 and gained an m-marker (arm-index about 1·6) which was a little longer than the chromosomes No. 16. Two of the hypomodal cells had lost two G chromosomes. The other two differed from the S-cells by loss of one C and one D chromosome, respectively. Ten of the fifteen 46-chromosome cells studied had a normal diploid karyotype and this population corresponded to an s. In the remaining five cells one chromosome Nos. 21–22 had bigger short arms and smaller long arms than normal (Fig. 1d, arrow). A pericentric inversion is a plausible explanation for the anomaly. However, in two of the cells the long arms of one chromosome No. 2 was about 15% longer than normal (Fig. 1d, arrow). Thus, more complex structural rearrangements must have occurred in at least a fraction of the cells with the G-anomaly.

*In M*5 all 45-chromosome cells studied had the same karyotype. The 2 cells with 46 chromosomes had also lost one G chromosome but there was one extra chromosome in group C. Conversely, the small s=44 showed a loss of one C chromosome, in addition to the loss in group G. In the other hypomodal cells the losses were restricted to groups C, D and G. In 4 of them there was a dicentric with a morphology varying from cell to cell. The only further structural deviation observed in this tumour was a probably acentric ring-chromosome in one of the cells with a dicentric.

*M*6. In this meningoma all 45-chromosome cells analyzed had lost 1 D chromosome (Fig. 2a). The hypomodal cells showed additional losses in group C and E, whereas the two cells with 2x=46 had a normal diploid karyotype. The near-triploid metaphase could not be analyzed. No marker was seen in any cell.

*M*7. In the first fixation, Pc-4, there were only cells with 45 chromosomes. Twelve of the 15 cells analyzed showed a loss of one chromosome Nos. 17–18 and one G chromosome and an added t-marker (Fig. 2b). The marker was intermediate in size between the smallest D and the biggest G chromosomes; the short arms often carried satellites indicating its probable origin from the G chromosome lost.

Two of the three variant cells differed from the S-cells by a loss of one F chromosome and an added st-marker. The marker had an arm-index of about 4·0 and its size was similar to that of the biggest C chromosomes. The remaining variant cell differed from the S-cells by a loss of the t-marker and 2 C chromosomes and addition of three new markers, one big, probably centric ring chromosome, one t-marker, half as big as the G chromosomes, and one sm-marker with an arm-index of about 3·5 and a size similar to that of the chromosomes No. 2.

In the passage, P7, the S was unchanged. All variant cells analyzed had the t-marker; those in the modal region showed additional deviations, usually losses, especially in groups B, C and D. Four of the hypomodal cells had one or two dicentrics with an inconsistent morphology. The polyploid elements accorded with doubling-products of modal and hypomodal cells. One of them had a t-marker, twice as big as the biggest D chromosomes.

*M*8. The last hypodiploid meningioma with S=45, had four markers in the stem line, M, st, $t_1$ and $t_2$ (Fig. 2c). The M-marker was a little smaller than the F chromosomes. The st-marker had an arm-index of about 3·6 and the size was similar to that of the bigger C chromosomes. The size of the minute $t_1$-marker was only about a third of that for the G chromosomes; the short arms sometimes seemed to carry satellites. The $t_2$-marker was about 25–30% longer than the biggest D chromosomes. The short arms were usually satellited. The morphology of the markers was similar in all cells.

In variant cells there were further losses in groups D and E and often also gains or losses in group C. In some cells one or two of the markers were missing and in a few others there was an additional t-marker, about half as big as the G chromosomes. The two hypotetraploid cells had a karyotype in accordance with doubling-products of S-cells.

The small and big acrocentrics had obviously participated in the formation of the t-markers. The origin of the other markers could not be traced.

*M*9. In this pseudodiploid meningioma all S-cells analyzed had the same karyotype (Fig. 2d). The s=44 and the exclusively hypomodal variant cells were closely related to the S and the gains and losses were restricted to groups C, D, F and G. Markers were absent in all cells studied.

*M*10. In the second pseudodiploid meningioma 18 of the 20 analyzed cells with the S-number had the same karyotype (Fig. 3a). One C chromosome was lost and an m-marker added; the marker had an arm-index of about 1·6 and the size was similar to that of the chromsomes No. 3. The other two cells with 46 chromosomes had the same karyotype as the S-cells except for one abnormal G chromosome which was about 30% shorter than the others. The single cell with 2x=47 differed from the S-cells by an added big t-marker, approximately twice as long as the smallest D chromosomes. The hypomodal cells showed additional losses in group A, C and D. The tetraploid cell could not be analyzed.

*M*11. In the third pseudodiploid meningioma 23 cells with the S-number were analyzed. Twenty-one of them had the same karyotype (Fig. 3b). The most obvious characteristics of the S was the loss of one C chromosome and the addition of 1 sm-marker. This marker had an arm-index of about 2·5 and a size similar to that of the chromosomes No 2. However, a closer examination revealed further structural deviations. Thus, two of the G chromosomes were approximately 30% shorter than normal and, in fact, represented t-markers. As in some variant cell of previous case (M10) the deviating G chromosomes looked deleted. In group C it was noticed that two or three biggest chromosomes consistently seemed to be abnormally big in relation to the other members of the group (Fig. 3b, arrows). The findings indicated that structural rearrangements had occurred in group C, though it was not possible to distinguish further markers. Translocations and deletions in groups C and G could account for the S-karyotype (as in previous tumour, M10).

The 2 deviating 46-chromosome cells differed from the S-cells by loss of one chromosome Nos. 17–18 and gain of one F chromosome. In the hypomodal cells there were additional losses in groups C, D and G. The three tetraploid cells had a karyotype corresponding to that of a doubled S-cell.

*M*12. The last meningioma had its stem line at the border between hyperdiploidy and hypotriploidy. The S=58, appearing in P1 and P2, differed from the original S=57 only by one further extra-chromosome in group C. Both of these closely allied stem lines had a t-marker (Fig. 3c); its size was intermediate between the smallest D and the biggest G chromosomes. The short arms of the t-marker often carried clear satellites. Thus, an acrocentric must have participated in its formation, though no loss was seen either in group D or group G. The t-marker was present in all analyzed hyperdiploid-hypotriploid variant cells from the first four fixations; as a rule these cells also showed deviations in the same chromosome groups as the S-cells. In variant cells with numbers just above or below the S-number there were only an increase or decrease of the extra C chromosomes. The cells with numbers between 46 and 54, however, showed a decrease or absence of the extra chromosomes also in groups B, D, E and F. The t-marker was also present in the three hypodiploid and the two diploid variant cells; in the hypodiploid cells one G and two to three C chromosomes were lost, whereas the 46-chromosome cells had lost only one G chromosome. The latter karyotype (+ one t-marker, − one G) was especially interesting since it characterized the S (Fig. 3d) of the two last fixations (P11 and P12). The 45-chromosome cells in P12 differed from the pseudodiploid S by loss of either one D or one F chromosome. The near-triploid cell had a karyotype similar to that seen in variant cells of the first four fixations.

The results illustrate an instance of differential growth rate of tumour cells *in vitro*. The findings also suggest that the tumour had had a pseudodiploid stem line during an earlier period of the *in vivo* growth. The t-marker was probably formed at that time and the loss in group G later on hidden by superimposed changes.

## DISCUSSION

The present study comprises 12 meningomas. The stem-sideline characteristics of an additional case, previously published [1], is shown in Table 3 (M13). The 13 tumours will

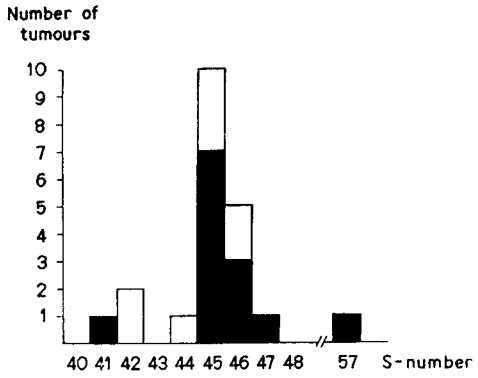


*Fig. 4. Stem line pattern in meningiomas (own material= blackened areas, Zang and Singer's material=open areas).*

be termed present material in the discussion, and the results considered in relation to findings in eight meningiomas reported by Zang and Singer [2]. All of their meningomas were studied in fixations from primary cultures with a duration of 4–9 days.

The stem line pattern in the 21 meningiomas is shown in Fig. 4. Almost half of the tumours had a 45-chromosome stem line and approximately one-quarter an S=46. Among the other 6 meningiomas 5 had near-diploid stem lines with predominance of S-numbers below 45. The preference for a hypodiploid evolution in these benign tumours is amply demonstrated by the fact that two-thirds of them have their S in the hypodiploid region; however, the cases with S=47 and S=57 illustrate that other progressional pathways do occur, though comparatively rarely. The findings in the present material agree with the results of Zang and Singer, but the peak at S=45 is less clear in their material and they also had more cases with S-numbers below 45.

Karyologically Zang and Singer found a loss of one G chromosome in 7 of their tumours; the eighth meningioma had a normal, diploid S but there was a small group of 45-chromosome cells with monosomy G. Among the seven tumours with abnormal stemlines the three cases with S=45 showed only a loss of one G-chromosome; the pseudodiploid and remaining three hypodiploid cases had stemlines with further deviations, usually losses, which engaged the chromosome groups C, D and E.

In the present material all of the 13 meningiomas had an abnormal S. Deviation in the G group occurred in 9 tumours and in 4 cases a loss of one G chromosome was the only abnormality of the S. Accordingly in both materials 7 tumours, one third of all cases, had a stem line with monosomy G as the only deviation from normal. Furthermore, among the 21 tumours 16 meningiomas or about three-quarters of the cases showed abnormalities in the group G. Thus, a preferential involvement of the small acrocentrics must be regarded as an established fact for this group of benign tumours.

Some observations indicate that the involvement of group G might be a secondary phenomenon related to progression. According to this idea the case (tumour T559, Zang and Singer [2]) with a normal diploid S and a small fraction of cells with monosomy G should represent an early stage in the progression and the cases with normal diploid sidelines or variant cells should illustrate later evolutionary steps; advanced stages in the progression should correspond to cases with additional losses in the G group and/or rearrangements in the other chromosome groups. Outgrowth of stroma cells could also account for the occurrence of normal diploid elements; this explanation, however, seems less likely due to the persistence of tumour stem lines in the three cases studied in fixations after 2–4 months' *in vitro* growth (M2, M7 and M12).

If the proposed theory of progression is accepted there would be four tumours left without engagement of group G: M6, M10, M12 and M13 (Table 3). This number can be reduced to three cases, since later fixations from M12 indicated that, at an earlier stage, it had possessed a pseudodiploid S with abnormalities engaging group G. In the remaining three cases small structural aberrations in group G might have past unnoticed. Thus, easily overlooked, small structural deviations in group G occurred in the S of M11 and were observed in variant cells in several other tumours of the present study. It is also possible that the engagement of group G is not an obligate karyotypic feature of the meningiomas. Thus, deviations in groups C or D could be alternatives to the affection of group G, an assumption supported by the observation that these two groups were predominantly engaged in cases with further abnormalities than monosomy G.

The representation of different chromosome types in the stem lines of the meningiomas is shown in Table 4. A statistical analysis has been carried out according to principles earlier outlined by Levan [8], and previously used in a study of malignant neurogenic tumours in children (last row, Table 4). Since no tumour with markers in its stem line occurred in Zang and Singer's material the present material was divided into two groups, meningiomas without and those with markers in their S. As in Zang and Singer's material those without markers showed a high negative value in group G

(less chromosomes than expected), a concordant but less prominent negative value in group D and a comparatively high positive value in group C; a slight difference was noticed in group F where the positive value was somewhat higher than in Zang and Singer's material. In the group of meningiomas with markers in their S the negative value in group G was considerably higher and (2nd row, Table 4) the positive value in group C was changed to a similar but negative value; the deviation in group D and F was no longer different from that observed in some other groups. These results suggest that in meningiomas with markers the chromosomes in group G and C participate in the formation of new chromosome types; and in the meningiomas without markers, both those of the present study and those in Zang and Singer's material, markers might be hidden in group C.

The occurrence of usually satellited t-markers in six of the seven tumours support this idea and indicates that the small acrocentrics (probably sometimes also the big acrocentrics) are frequently involved in structural rearrangements. In this context it should be mentioned that the acrocentrics are known to be more unstable, both numerically and structurally, than any other chromosome types in the human complement (for review, see Levan [8] ).

Zang and Singer stated that in their eight meningomas, "there were no essential structural abnormalities in any chromosomes"; in a later survey of cytogenetics of human tumours [3] they re-emphasized that structural aberrations were rare in the meningomas studied by them and they briefly referred to analyses of more than 30 cases. In the present material there were markers in more than 50% of the stem lines, and more than one marker occurred in about 40% of these cases. Furthermore, especially the small acrocentrics and also group C seemed to be preferentially involved in the structural rearrangements. No satisfactory explanation can be offered for this discrepancy, between the present results and those reported by Zang and Singer.

Zang and Singer also reported a uniform chromosome set in samples of all tumours studied by them and no case with "great variations from cell to cell as described for malignant tumours" [3]. This was true for most tumours in the present material but some cases as M1 and M12 showed a karyotypic variation comparable to that seen in malignant human tumours [4]. This question was elucidated further by a comparison of the chromosomal deviations in stem lines of meningiomas with the correspondent deviations seen in a group of primary malignant human tumours. The latter material consisted of 22 malignant neurogenic tumours in children [4]. The mean differences found or expected was calculated for this group (last row, Table 4). As in the meningiomas significant deviations occurred only in groups C, D, G and markers. However, in the malignant tumours the positive value in group C was higher than in the meningiomas, and the negative value in group D was equal to that in group G, whereas the positive value for markers was the same in both tumour groups. The results suggest that similar mechanisms for chromosomal variation are operating in both benign and malignant tumours; however, the results also indicate that some of these mechanisms might predominate in a certain tumour group, benign or malignant.

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## SUMMARY

*The chromosomes of 12 human meningiomas have been studied. All of them had an abnormal stem line. The majority of the tumours had a hypodiploid stem line, and there was a peak at S=45. Karyotype analyses demonstrated numerical and/or structural deviations in group G in about three quarters of the tumours. According to findings in the present and a previous study the preferential involvement of group G might be a phenomenon related to progression. Conversely to a previous study the present investigation showed markers in the stem lines of about half of the tumours. A statistical analysis of the chromosomal representation in the tumour stem lines indicated that the groups G and C were involved in the formation of markers, and that markers could be hidden in group C in cases without discernible structural deviations. Furthermore, in the meningiomas and in a group of malignant primary tumours (neurogenic tumours in children) the major deviations appeared to be restricted to the same chromosome groups. Thus, similar mechanisms for chromosomal variation seem to be operating in both benign and malignant human tumours.*

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# Transplantation and Tissue-Culture Studies of Canine Osteosarcoma

D. E. BOSTOCK and L. N. OWEN
*Department of Animal Pathology, University of Cambridge, Great Britain*

## INTRODUCTION

A DESCRIPTION of the transplantation of a canine osteosarcoma by the injection of tumour cells into canine foetuses *in utero* has been previously given [1]. In the present experiments the establishment of the 4th transplant generation of this tumour in tissue culture is described.

## MATERIAL AND METHODS

*Tissue culture*

The original osteosarcoma was derived from an 8 year-old cross-bred Alsatian with a spontaneously arising tumour of the proximal end of the left radius. Tumour from the 4th passage in a 3 month-old female Retriever puppy was removed surgically, diced in Hank's solution and tryspinised at 38°C in the usual manner using 0·25% trypsin for 20 min. After centrifugation the cells were resuspended in tissue culture medium to give a concentration of $1 \cdot 25 \times 10^6$ cells/ml. The tissue culture media used were TC199, Hank's medium or Eagle's medium, each medium containing also 20% foetal calf serum.

*Histochemistry*

Histochemical studies were made on tumour cells growing *in vitro* as monolayers and *in vivo* in puppies by Dr. G. M. Jeffree by the method of Jeffree and Price [2].

*Growth of tissue-culture cells in puppies*

Tissue-culture cells grown in TC199 containing 20% foetal calf serum were injected into:

(1) Canine foetuses *in utero*,
(2) Puppies given 200 r whole-body irradiation a few hours after birth,
(3) One newborn puppy given 5 mg/kg azathioprine orally daily,
(4) Newborn puppies treated with anti-lymphocyte serum (A.L.S.),
(5) Normal newborn puppies.

Anti-lymphocyte serum was prepared from rabbits which had been twice injected intravenously with a cell suspension of thymus, mesenteric, and internal iliac lymph nodes obtained from dogs 2–3 months old. The interval between injections was 3 weeks. The rabbits were bled out 4 weeks after the first injection and the serum was absorbed on normal canine red blood cells but not otherwise purified.

The dose for puppies was about the same as that used by Simpson [3], i.e. 2 ml administered subcutaneously 3 times weekly, but counts were made on blood lymphocytes and the dose reduced when necessary.

## RESULTS

*Growth and morphology of tumour cells in tissue culture*

The tumour cells grew only in TC199+20% foetal calf serum. Growth was rapid and the cells which had piled up on the glass after 48 hr were versenated and re-seeded in fresh medium. After 24 hr growth; long fibroblastic cells arranged singly were visible on the glass and by 72 hr a complete monolayer of fibroblastic cells arranged in whorls and bundles was present (Fig. 1). The cells continued to grow with the same morphology through many passages. The doubling time was about 72 hr

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and the cell line has now reached the 65th passage. Later passages grew well in TC199 with the addition of 10% foetal calf serum, but not in Hank's medium.

Similar cell lines showing identical morphological characteristics have been obtained from the 5th and 6th *in vivo* transplantations of the original tumour and have reached their 35th and 11th passages respectively. As well as fibroblastic cells with oval nuclei containing several prominent nucleoli true giant cells have been occasionally seen. Tissue culture cells treated with dimethyl sulphoxide and frozen in liquid nitrogen remained viable. There was, however, a time-lag up to 2 weeks before normal growth was established when these cells were again transferred to tissue culture medium.

The tissue culture cells at the 25th and 50th passage levels contained the normal number of chromosomes for the dog (78) and had a chromosome complement characteristic for the male. This shows that the cells were derived from the original male donor rather than the 4th *in vivo* passage host which was female.

*Histochemistry*

Enzyme studies on 4th *in vivo* transplanted tumours showed the high alkaline phosphatase activity normally seen in osteosarcomas. This was maintained in tissue culture and also in tumour grown in newborn puppies immunosuppressed with anti-lymphocyte serum. In contrast, tissue culture cells grown from 5th *in vivo* transplanted tumour were almost completely negative for alkaline phosphatase.

Acid phosphatase, beta-glucuronidase, lactic acid dehydrogenase and non-specific esterase activity present in the 4th *in vivo* transplant tumour were also found in tissue culture cells but appeared to be decreased in the case of the first two enzymes.

*Establishment of tumours in puppies from tissue-culture cells*

Only in puppies either injected as foetuses *in utero* or newborn puppies treated with anti-lymphocyte serum was there progressive tumour growth with death of the animal (Table 1). In one normal puppy injected at birth there was progressive tumour growth for 3 weeks followed by regression of the tumour.

The most successful experiment using anti-lymphocyte serum was one in which newborn puppies were injected with $10^8$ cells from the 44th tissue culture passage (4th *in vivo* transplant). Two puppies were injected subcutaneously and one puppy both intravenously and subcutaneously. This last puppy developed nystagmus and dyspnoea when 8 weeks old and was killed 2 weeks later. Radiographic examination showed widespread circular osteolytic lesions affecting both membrane and cartilage bone and most noticeable in the long bones of the limbs, the ribs and the skull (Figs. 2 and 3). The metatarsals, metacarpals and phalanges were unaffected.

Post-mortem examination revealed massive tumour deposits in the bones, lungs and meninges (Fig. 4) and at the site of the subcutaneous injection in the neck.

The tumour from lung has been re-established in tissue culture and the cells have a similar morphology to the original tissue culture cells.

One puppy injected subcutaneously produced tumours at the site of inoculation and also in the lungs, kidneys, petrous temporal bone and the gum over the right maxillary canine tooth. Tumours in the gum have been previously described [1]. The second puppy grew a subcutaneous tumour only, which regressed after 5 months in spite of continued treatment with anti-lymphocyte serum. Tumours from both these animals were histologically similar to those seen in the puppy injected intravenously.

A normal litter-mate of these 3 puppies (i.e. not injected with A.L.S.) was also injected with $10^8$ tumour cells subcutaneously but no tumour growth occurred.

*Table* 1. *Results of inoculation of foetal and neonatal dogs with tissue-culture cells*

| Treatment | No. of dogs treated | No. of tumour cells injected | No. of dogs killed by tumour | Length of time to death in days |
|---|---|---|---|---|
| Injected *in utero* | 11 | $10^7$–$5\times10^7$ | 8 | 35, 50, 55, 71, 73, 85, 88, 93 |
| 200 r whole-body irradiation | 4 | $5\times10^7$ | 0 | — |
| 5 mg/kg Azothioprine daily | 1 | $10^8$ | 0 | — |
| Newborn puppies treated with anti-lymphocyte serum | 9 | $10^8$ | 5 | 33, 45, 81, 85, 86 |
| Newborn puppies without treatment | 11 | $10^8$ | 0 | — |

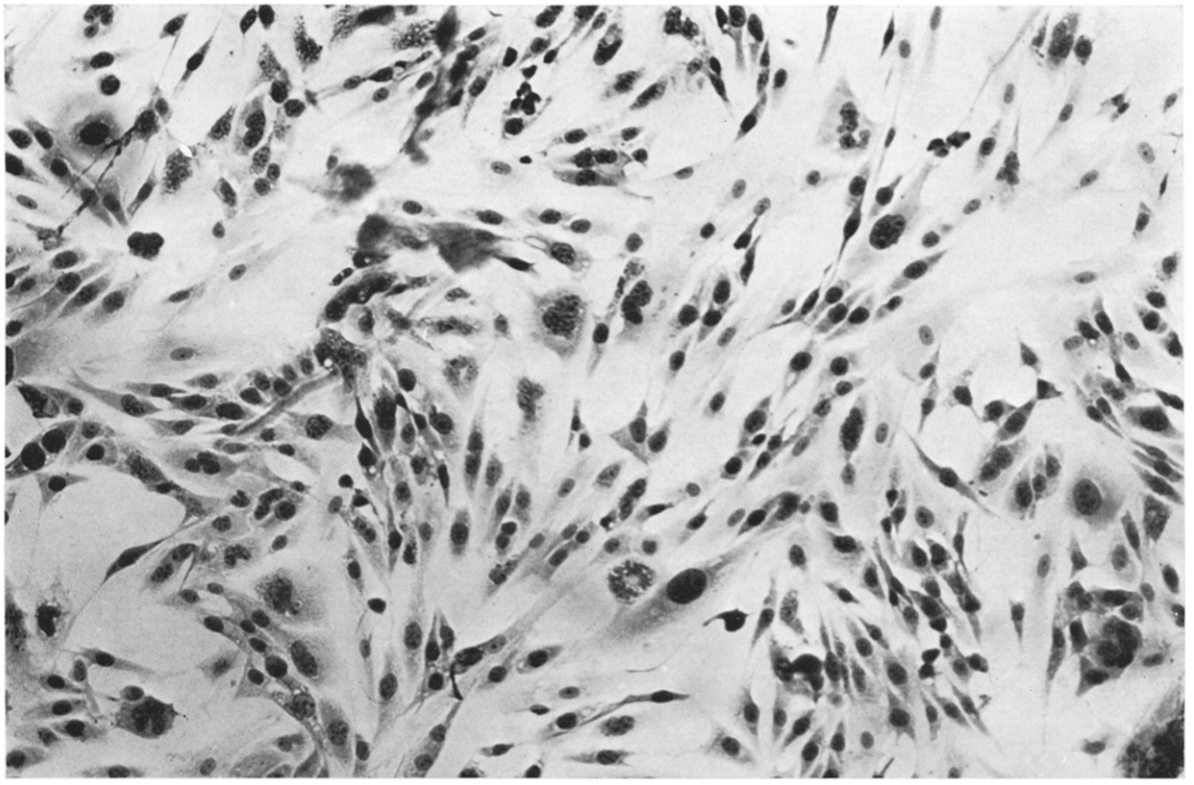

*Fig.* 1. *Appearance of osteosarcoma cells in tissue culture. Thirtieth* in vitro *passage cultured for* 3 *days. Giemsa.* ×250.

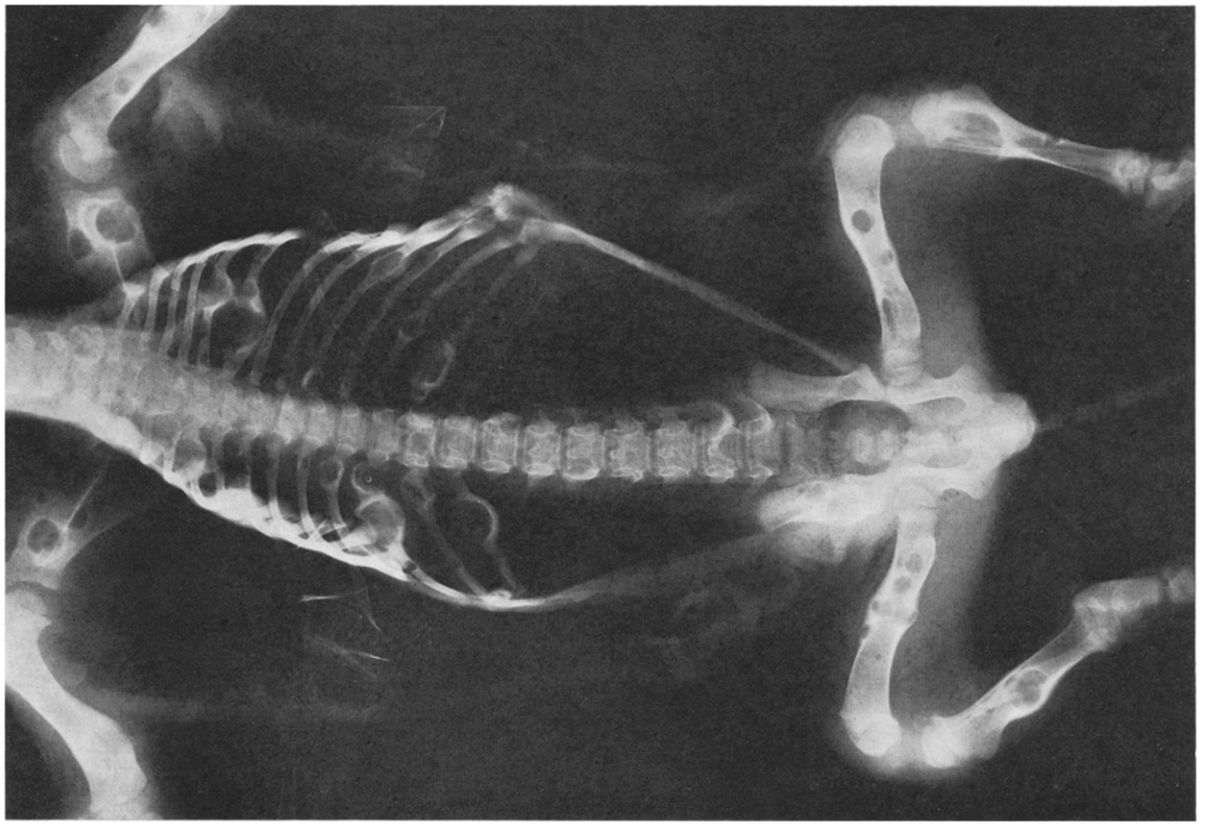

*Fig.* 2. *Widespread osteolytic tumours in bone following the intravenous injection of tissue-culture cells* 8 *weeks previously. The puppy was immunosuppressed with anti-lymphocyte serum.*

(to face p. 500)

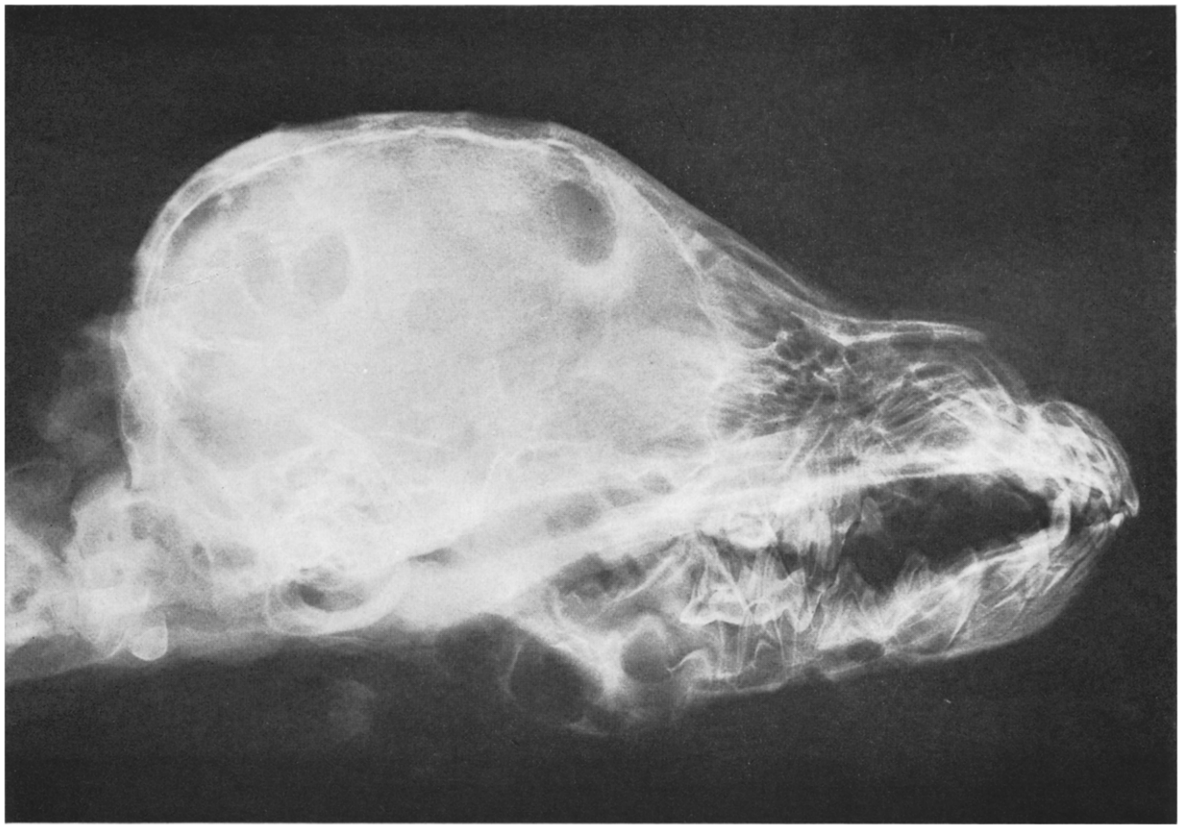

*Fig.* 3. *The same case as Fig.* 2 *showing osteolytic lesions in the skull.*

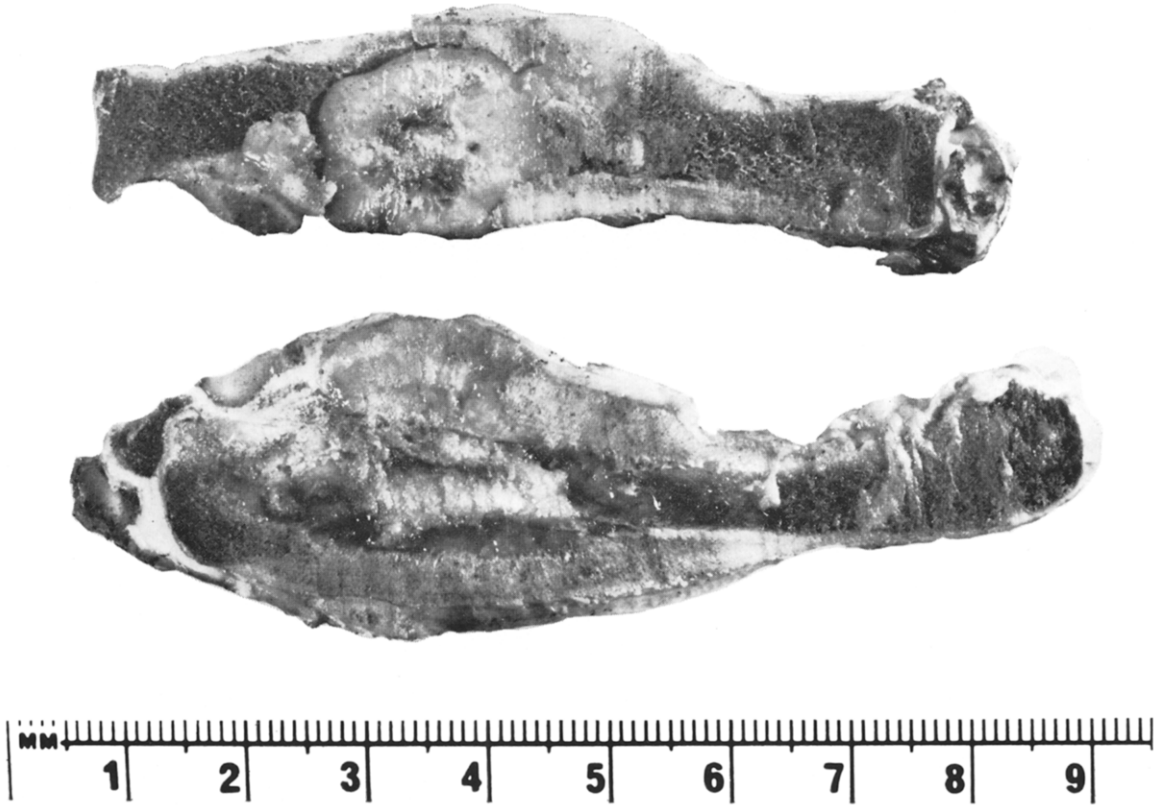


*Fig.* 4. *Post-mortem appearance of bone tumours in femur and tibia. (Radiographic appearance shown in Fig.* 2.)

One of two Wire-Haired Fox Terriers injected intra-peritoneally with $10^7$ tissue culture cells, when 45-day-old foetuses, developed multiple and widespread tumours of the bones of similar appearance to those found in the A.L.S.-treated puppy, and died when 10 weeks old.

Four Alsatian puppies were injected intraperitoneally with $2 \times 10^7$ tissue culture cells when 46-day-old foetuses. Three of these puppies were injected with $3 \times 10^7$ cells intravenously on the day of birth, and these animals developed various tumours a few weeks later. The first dog clinically affected had tumours in the lungs, heart, diaphragm, kidneys, spleen and mesenteric lymph nodes. In the second affected animal there were tumours in lungs and heart only, but in the third animal the distribution was again widespread and included tumours in the sub-cutis, gum and left proximal humerus. The fourth animal which was injected subcutaneously at birth with $3 \times 10^7$ cells developed subcutaneous tumours which later regressed.

Histological examination of these various tumours has shown in most areas closely packed spindle-shaped cells with little intercellular matrix. In some areas there were round cells and the tumour was rather more pleomorphic and yet other areas showed a fibro-spindle cell sarcoma which was well collagenised.

## DISCUSSION

There are only few reports of growth of canine tumours in tissue culture. Cultivation of malignant cells from a canine Sertoli cell adeno-carcinoma was achieved by Rosanoff [4]. At the time of the report the cell line had undergone 55 passages. Following inoculation of these cells into 2 4-month-old dogs treated with Metacortelone acetate tumours were produced but no details are given of progression or regression of these tumours.

Canine thyroid adenocarcinoma and canine melanoma cell lines have been established by Kasza [4] and maintained for more than five years [5]. Melanoma cells inoculated into young dogs produced a tumour which could kill the animals within 8 weeks.

Simpson [3] has grown a canine sarcoma *in vitro* and successfully transplanted it into newborn puppies immunosuppressed with anti-lymphocyte serum. We have attempted to establish tissue culture lines from 8 spontaneous osteosarcomas in dogs but only in the osteosarcoma transplant described in this article were we successful.

In puppies injected with tissue culture cells the growth of a more spindle cell sarcomatous type of tumour as compared with the original osteosarcoma is not unexpected. Other workers using rats and mice have found similar histological changes following tumour transplantation. However, Stewart *et al.* [7] were able to produce two sublines of transplanted tumour in C3H mice, one with bone and the other with little bone.

Metastasis of osteosarcoma to bone in man occurs in about 25% of cases but, perhaps because of early euthanasia, appears to be much less than this in spontaneous osteosarcoma in the dog. Suter [8] gives a figure of only 4%. The remarkable distribution of tumours in bone following the intravenous injection of tissue culture cells into a puppy treated with A.L.S. which was also seen in puppies injected intraperitoneally when foetuses *in utero* and the mechanisms of bone destruction itself are receiving further study. Unlike some of the tumours which overlay the ribs in previously described cases of transplanted osteosarcoma in dogs [1] the present tumours developing from tissue culture cells were intramedullary in the long bones and had also destroyed large areas of membrane bone.

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## SUMMARY

*A canine osteosarcoma, which was transplanted by the inoculation of trypsinised cells into dog foetuses* in utero, *has been grown in tissue culture. These tissue-culture cells, when inoculated into canine foetuses* in utero, *produced spindle cell sarcomas, which frequently affected the bones. Similar results were obtained when the tissue culture-cells were inoculated into new born puppies which were immunosuppressed by anti-lymphocyte serum.*

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# Subcellular Particles in Tumors—IV Lysosomes in Hepatoma HC and Morris Hepatomas 7794A, 7794B, 5123A, 7316A and 16

R. WATTIAUX*, S. WATTIAUX-DE CONINCK*, J. M. VAN DIJCK*, M. F. DUPAL* and H. P. MORRIS†

**Laboratory of Physiological Chemistry, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium; and †Department of Biochemistry, Cancer Research Unit, College of Medicine, Howard University, Washington, D.C.*

## INTRODUCTION

LYSOSOMES are cytoplasmic particles containing numerous acid hydrolases of various specificities. They take part in the intracellular digestive processes involving exogenous material taken up by the cell as well as endogenous components during autophagy [1]. Their presence in hepatomas has been ascertained by morphological observation and biochemical studies [2–5].

The present work is a relatively detailed investigation of the properties of these granules in several transplantable hepatomas: 1 rapidly growing tumour (HC hepatoma), 4 medium- to slowly-growing tumors (Morris hepatomas 7794A, 7794B, 5123A, 7316A), and 1 slowly growing tumor (Morris hepatoma 16). The activity of 7 acid hydrolases was measured in tumor homogenates and their distribution established after differential centrifugation. Of these acid hydrolases, 3 were chosen as reference enzymes to characterize the behaviour of lysosomes in density gradients. In addition, granule stability *in vitro* was investigated making use of acid phosphatase as a marker enzyme. The properties of hepatoma lysosomes were inferred from the results and compared with those of rat liver organelles.

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## MATERIAL AND METHODS

*Animals*

Morris hepatomas 7794B (generations 13, 14, 15 and 16), 7794A (generations 26, 27 and 29), 5123A (generations 79, 80 and 84), 7316A (generations 33, 34, 35 and 36), 16 (generation 3) and HC hepatoma (generations 145 and 151) were transplanted into Buffalo strain rats at Howard University, Washington, D.C.; the tumor-bearing animals were shipped by air express to our laboratory in Namur, Belgium. The average times in days between transplantation and collecting of the tumors were: $76\pm13$ for 7794B, $98\pm17$ for 7794A, $43\pm6$ for 5123A, $60\pm8$ for 7316A, $176\pm8$ for 16 and $22\pm3$ for HC.

*Tissue fractionation*

The rats were killed by decapitation. The tumor was rapidly dissected, cut in small pieces on a refrigerated Petri dish and placed in ice-cold 0·25 M sucrose. The tumor was homogenized in the same medium by means of a smooth glass tube fitted with a Teflon pestle rotating at 3000 rev/min. The suspension was brought to a volume of approximately 40 ml and spun for 10 min at 1500 rev/min (470 *g*

*Table* 1. *Intracellular distribution of enzymes*

*Absolute values are given in mg/g for proteins and in unit/g fresh weight of tumor for enzymes. The results are given as means ± SD.E, cytoplasmic extract; N, nuclear fraction M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant.*

| Enzyme | No. of expts. | Absolute values E+N | Percentage values | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | E+N | N | M | L | P | S | Recovery |
| Hepatoma HC | | | | | | | | | |
| Proteins | 3 | 143±9 | 100 | 29·8±0·7 | 14·8±0·8 | 2·9±0·1 | 17·3±0·6 | 37·1±3·4 | 101·9± 3·3 |
| Acid ribonuclease | 3 | 0·54±0·04 | 100 | 23·2±4·7 | 23·1±1·5 | 12·9±1·5 | 11·5±0·6 | 16·4±3·8 | 87·1± 3·4 |
| Acid deoxyribonuclease | 3 | 0·57±0·06 | 100 | 23·3±2·5 | 26·2±3·5 | 16·9±2·8 | 14·2±0·7 | 19·7±4·4 | 100·3± 3·6 |
| Acid phosphatase | 3 | 3·34±0·20 | 100 | 23·4±4·3 | 28·6±3·0 | 18·7±2·0 | 21·0±1·5 | 13·8±2·3 | 105·5± 6·6 |
| β.N.acetylglucosaminidase | 3 | 3·40±0·79 | 100 | 25·1±1·0 | 34·3±0·2 | 18·7±2·2 | 18·0±1·0 | 7·1±1·8 | 103·2± 0·9 |
| β.galactosidase | 3 | 0·43±0·05 | 100 | 24·2±4·6 | 30·0±2·5 | 16·1±1·0 | 7·5±0·3 | 23·3±4·3 | 101·1± 7·0 |
| Cathepsin | 3 | 0·40±0·05 | 100 | 22·3±2·3 | 33·4±2·2 | 20·6±3·0 | 9·3±0·1 | 11·8±3·5 | 97·4± 5·3 |
| Acid exonuclease | 3 | 0·46±0·09 | 100 | 20·3±1·3 | 31·0±3·2 | 20·2±2·7 | 11·3±1·6 | 14·3±3·4 | 97·1± 0·6 |
| Hepatoma 7794A | | | | | | | | | |
| Proteins | 4 | 169±8 | 100 | 28·0±2·5 | 13·3±2·2 | 44·5±0·7 | 12·8±1·1 | 38·0±0·4 | 97·1± 2·3 |
| Acid ribonuclease | 4 | 0·99±0·11 | 100 | 30·3±5·6 | 27·8±1·7 | 16·8±2·5 | 10·1±2·3 | 13·3±6·4 | 98·3± 2·3 |
| Acid deoxyribonuclease | 4 | 0·62±0·04 | 100 | 30·4±2·0 | 26·0±3·4 | 17·4±2·6 | 15·2±2·8 | 12·5±2·9 | 101·5±10·3 |
| Acid phosphatase | 4 | 4·63±0·52 | 100 | 25·1±4·1 | 30·4±5·8 | 25·0±2·3 | 13·1±1·5 | 6·8±1·3 | 100·4± 4·9 |
| β.N.acetylglucosaminidase | 4 | 3·11±0·95 | 100 | 22·5±5·2 | 33·6±0·7 | 29·3±3·2 | 9·9±2·1 | 3·9±1·8 | 99·2± 2·7 |
| β.galactosidase | 4 | 0·58±0·06 | 100 | 29·3±3·1 | 32·3±1·7 | 20·4±1·9 | 4·9±1·7 | 12·6±4·9 | 99·5± 3·4 |
| Cathepsin | 4 | 0·67±0·07 | 100 | 34·3±5·9 | 33·8±1·3 | 25·5±1·6 | 7·4±1·2 | 5·5±3·6 | 106·5± 2·5 |
| Acid exonuclease | 4 | 0·70±0·11 | 100 | 28·7±4·8 | 30·4±3·3 | 17·9±1·1 | 7·9±1·0 | 10·2±3·3 | 95·1± 6·6 |
| Hepatoma 7794B | | | | | | | | | |
| Proteins | 4 | 191±8 | 100 | 38·5±2·2 | 16·3±1·7 | 2·9±0·3 | 10·2±1·2 | 30·6±4·2 | 98·5± 2·1 |
| Acid ribonuclease | 4 | 0·60±0·10 | 100 | 29·6±2·4 | 22·7±0·5 | 15·4±3·0 | 9·9±2·6 | 21·1±5·2 | 98·7± 5·7 |
| Acid deoxyribonuclease | 4 | 0·37±0·07 | 100 | 31·6±4·2 | 27·6±2·2 | 15·9±2·2 | 15·7±2·1 | 20·1±1·4 | 110·9± 1·8 |
| Acid phosphatase | 4 | 3·82±0·80 | 100 | 29·8±2·6 | 25·6±2·4 | 26·4±3·2 | 13·4±3·1 | 10·4±2·5 | 105·6± 3·8 |
| β.N.acetylglucosaminidase | 4 | 1·24±0·49 | 100 | 29·8±5·7 | 28·0±1·6 | 23·1±4·1 | 12·3±2·0 | 8·1±4·9 | 101·3± 4·6 |
| β.galactosidase | 4 | 0·35±0·04 | 100 | 30·2±2·5 | 23·9±1·7 | 20·2±2·3 | 6·7±1·3 | 15·1±3·8 | 96·1± 3·3 |
| Cathepsin | 4 | 0·60±0·07 | 100 | 33·0±2·1 | 31·6±3·6 | 21·3±1·8 | 7·5±1·6 | 11·0±2·9 | 104·4± 1·8 |
| Acid exonuclease | 4 | 0·67±0·03 | 100 | 26·6±3·4 | 28·4±2·0 | 15·0±2·7 | 7·9±1·3 | 15·8±2·4 | 93·7± 2·1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Hepatoma 7316A | | | | | | | | | |
| Proteins | 3 | 184±7 | 100 | 29·1±2·4 | 21·8±2·6 | 4·0±0·8 | 13·4±0·4 | 30·3±0·9 | 98·6± 1·9 |
| Acid ribonuclease | 3 | 1·09±0·21 | 100 | 26·6±4·1 | 24·8±4·2 | 18·9±4·6 | 9·4±0·7 | 15·9±2·3 | 95·6± 4·9 |
| Acid deoxyribonuclease | 3 | 0·83±0·01 | 100 | 31·0±3·4 | 26·4±1·6 | 18·8±3·1 | 14·7±0·1 | 11·4±1·6 | 102·3± 3·5 |
| Acid phosphatase | 3 | 5·27±0·57 | 100 | 22·5±5·4 | 33·1±9·0 | 29·3±6·2 | 12·4±0·3 | 11·0±0·4 | 108·3± 9·5 |
| β.N.acetylglucosaminidase | 3 | 2·15±0·07 | 100 | 28·3±2·3 | 26·3±1·4 | 26·6±5·2 | 12·1±0·4 | 5·0±1·6 | 98·3± 4·9 |
| β.galactosidase | 3 | 0·73±0·13 | 100 | 28·2±3·3 | 30·1±2·5 | 20·9±2·3 | 6·0±0·7 | 15·5±1·4 | 100·7± 3·1 |
| Cathepsin | 3 | 0·81±0·16 | 100 | 33·4±3·2 | 32·5±4·5 | 23·7±3·4 | 7·6±0·6 | 8·0±1·0 | 105·2± 5·3 |
| Acid exonuclease | 2 | 0·93±0·14 | 100 | 24·8±6·5 | 30·5±4·5 | 19·5±4·9 | 6·9±0·8 | 11·1±0·2 | 92·8± 3·9 |
| Hepatoma 5123A | | | | | | | | | |
| Proteins | 4 | 150±8 | 100 | 30·5±2·9 | 15·3±0·7 | 5·0±0·4 | 12·4±1·8 | 35·1±1·3 | 98·3± 0·7 |
| Acid ribonuclease | 4 | 1·06±0·10 | 100 | 18·8±3·3 | 27·2±2·8 | 20·8±4·2 | 12·5±2·2 | 21·0±9·6 | 100·3± 5·8 |
| Acid deoxyribonuclease | 4 | 0·86±0·12 | 100 | 20·0±2·2 | 34·4±1·9 | 22·3±4·0 | 13·8±0·8 | 14·6±9·7 | 105·1± 2·8 |
| Acid phosphatase | 4 | 10·10±1·75 | 100 | 16·0±1·9 | 31·6±1·0 | 25·7±4·6 | 12·9±1·5 | 10·9±4·3 | 97·1± 3·6 |
| β.N.acetylglucosaminidase | 4 | 8·93±2·20 | 100 | 19·2±2·2 | 33·8±3·1 | 29·2±6·1 | 15·7±1·2 | 4·3±3·1 | 102·2± 1·5 |
| β.galactosidase | 4 | 1·17±0·30 | 100 | 19·5±2·6 | 32·5±2·2 | 23·3±5·5 | 8·4±0·8 | 16·3±7·4 | 100·0± 2·0 |
| Cathepsin | 4 | 0·70±0·13 | 100 | 19·4±3·4 | 41·1±3·3 | 24·0±3·3 | 8·5±1·9 | 10·1±7·2 | 103·1± 4·6 |
| Acid exonuclease | 4 | 1·08±0·46 | 100 | 20·6±5·9 | 36·1±4·0 | 18·9±2·7 | 8·7±2·0 | 14·8±4·8 | 99·1± 9·5 |
| Hepatoma 16 | | | | | | | | | |
| Proteins | 3 | 162+17 | 100 | 12·2+1·6 | 15·1+0·8 | 13·7+2·8 | 18·8+1·1 | 37·7+1·1 | 97·5+ 2·6 |
| Acid ribonuclease | 3 | 0·78+0·14 | 100 | 10·1+2·3 | 36·6+3·9 | 22·6+3·4 | 14·0+1·7 | 17·4+5·7 | 100·7+ 9·0 |
| Acid deoxyribonuclease | 3 | 0·65+0·11 | 100 | 11·6+2·0 | 41·7+3·1 | 23·7+1·4 | 13·7+3·6 | 15·4+2·5 | 106·1+ 6·0 |
| Acid phosphatase | 3 | 4·76+0·75 | 100 | 5·9+1·0 | 43·6+4·4 | 33·0+3·5 | 13·5+2·9 | 8·3+3·5 | 104·3+ 7·5 |
| β.N.acetylglucosaminidase | 3 | 2·12+0·47 | 100 | 9·2+1·7 | 40·5+2·1 | 24·7+2·5 | 15·4+4·2 | 5·1+1·0 | 94·9+ 3·6 |
| β.galactosidase | 3 | 0·29+0·05 | 100 | 11·3+0·9 | 40·0+5·8 | 21·9+3·4 | 6·9+1·4 | 25·0+7·1 | 105·1+ 1·8 |
| Cathepsin | 3 | 0·93+0·20 | 100 | 8·6+1·8 | 55·3+2·2 | 29·6+1·3 | 8·3+3·2 | 7·3+2·2 | 109·1+ 6·5 |

average) in an International $PR_2$ refrigerated centrifuge. The sediment was washed twice in the same conditions. Supernatants obtained from the centrifugations were pooled to make the cytoplasmic extract E. Fractionation of this extract was achieved as previously described [4, 6] using the technique of de Duve *et al.* [7]. Density gradient centrifugation experiments were performed according to Beaufay *et al.* [8].

*Enzymes assays*

Acid phosphatase, acid ribonuclease, acid deoxyribonuclease and cathepsin were assayed by the procedure of Wattiaux and de Duve [9], with the exception that for deoxyribonuclease the incubation medium contained 0·2 M KCl. β-Galactosidase and N-acetylglucosaminidase were measured according to Vaes [10] using *p*-nitrophenyl-β-D-galactoside and p-nitrophenyl-N-acetyl-β-D-glucosaminide respectively as substrates. Acid exonuclease was measured by the method of Carrara and Bernardi [11], with "DNA core" as the substrate obtained by digestion of thymus DNA by purified DNase II. Proteins were assayed by the method of Lowry *et al.* [12], utilizing bovine serum albumin as standard.

For acid phosphatase, N-acetylglucosaminidase, and β-galactosidase the unit of enzyme activity was defined as the amount of enzyme causing the decomposition of 1 μmole of substrate/min under the conditions of the assay. The breakdown of DNA, RNA and "DNA core" was calculated in mononucleotides: one unit of nuclease activity corresponded to the amount of enzyme causing the liberation of 1 μmole of nucleotide/min. The molarity of the products of cathepsin action was expressed in terms of tyrosine equivalents as shown by the colour developed with the Folin Ciocalteu reagent.

## RESULTS

*Activity of acid hydrolases and distribution after differential centrifugation*

Listed in the third column of Table 1 are the mean activities of the acid hydrolases in hepatoma homogenates. Obviously, the 6 hepatomas possess a readily measurable activity and significant differences exist among the tumors. Figure 1 permits a comparison between the specific activities observed and those found in the rat liver homogenate, enzymatic tests being performed in the same conditions. As a rule, the enzymes in the tumors exhibit a specific activity ranging from 50 to 150% of that found in rat liver tissue. There is, however, a major exception: the specific activities of acid phosphatase, N-acetylglucosaminidase and β-galactodidase of hepa-

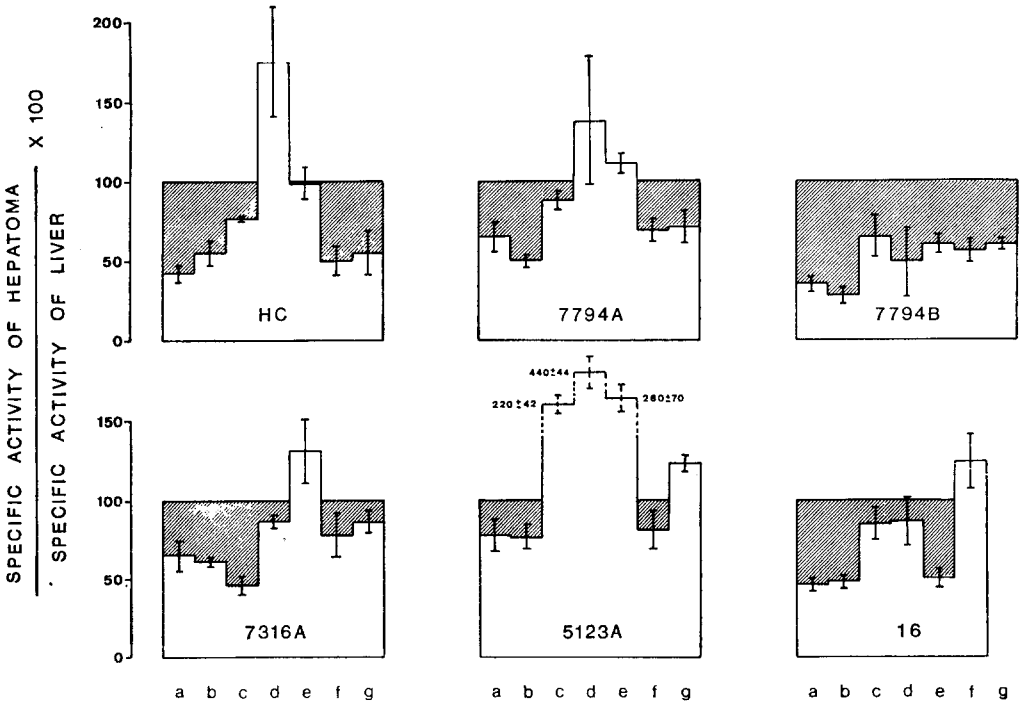


*Fig. 1. Comparison between the specific activity (units/mg of protein) of acid hydrolases in hepatoma and rat liver homogenates. The shaded area allows to visualize the differences between tumors and liver. The results are presented as means ±S.D. (a) acid RNase; (b) acid DNase; (c) acid phosphatase; (d) N-acetylglucosaminidase; (e) β-galactosidase; (f) cathepsin; (g) acid exonuclease.*

toma 5123A are far greater than those found in liver homogenates.

The mean values for distributions obtained after differential centrifugation are shown in Table 1 while the corrected distribution patterns are illustrated in Fig. 2 in the manner proposed by de Duve *et al.* [7]. The same general distribution pattern characterizes the acid hydrolases of hepatomas HC, 7794A, 7794B, 7316A and 5123A. The enzymes are found mainly in the mitochondrial fractions and exhibit a peak of specific activity in the light mitochondrial fraction. A significant proportion of the enzymes is also recovered in the soluble fraction. This distribution pattern resembles strikingly the distribution of the same enzymes in rat liver [7]. Acid hydrolases of hepatoma 16 are also chiefly associated with the mitochondrial fraction. However, the distribution pattern recorded in Fig. 2 differs from that of acid hydrolases of the other hepatomas in that no specific activity peak is observed in the light mitochondrial fraction L. As shown in Table 1, the distribution of the hydrolases in the two mitochondrial fractions is analogous to that found in other hepatomas but both the light and heavy mitochondrial fractions contain a similar amount of protein. Indeed, there is equal recovery of hepatoma 16 mitochondria in the two mitochondrial fractions M and L (results to be published); thus, the light mitochondrial fraction L contains much more mitochondrial protein than the L fraction of other hepatomas or liver, which significantly lowers the specific activity of acid hydrolases recovered in that fraction.

*Distribution after isopycnic centrifugation*

Distributions were established after centrifugation in a sucrose gradient and in gradients consisting of glycogen dissolved in a sucrose solution of known molality. Experiments were usually performed in 0–20% glycogen gradients with 0·204, 0·561, 0·988 and 1·470 molal sucrose in water as solvent. The average recoveries of enzymes for all the experiments were 100·3%±11·1 (33) for acid ribonuclease, 102·1%±10·6 (33) for acid deoxyribonuclease and 102·1%±9·4 (35) for acid phosphatase.

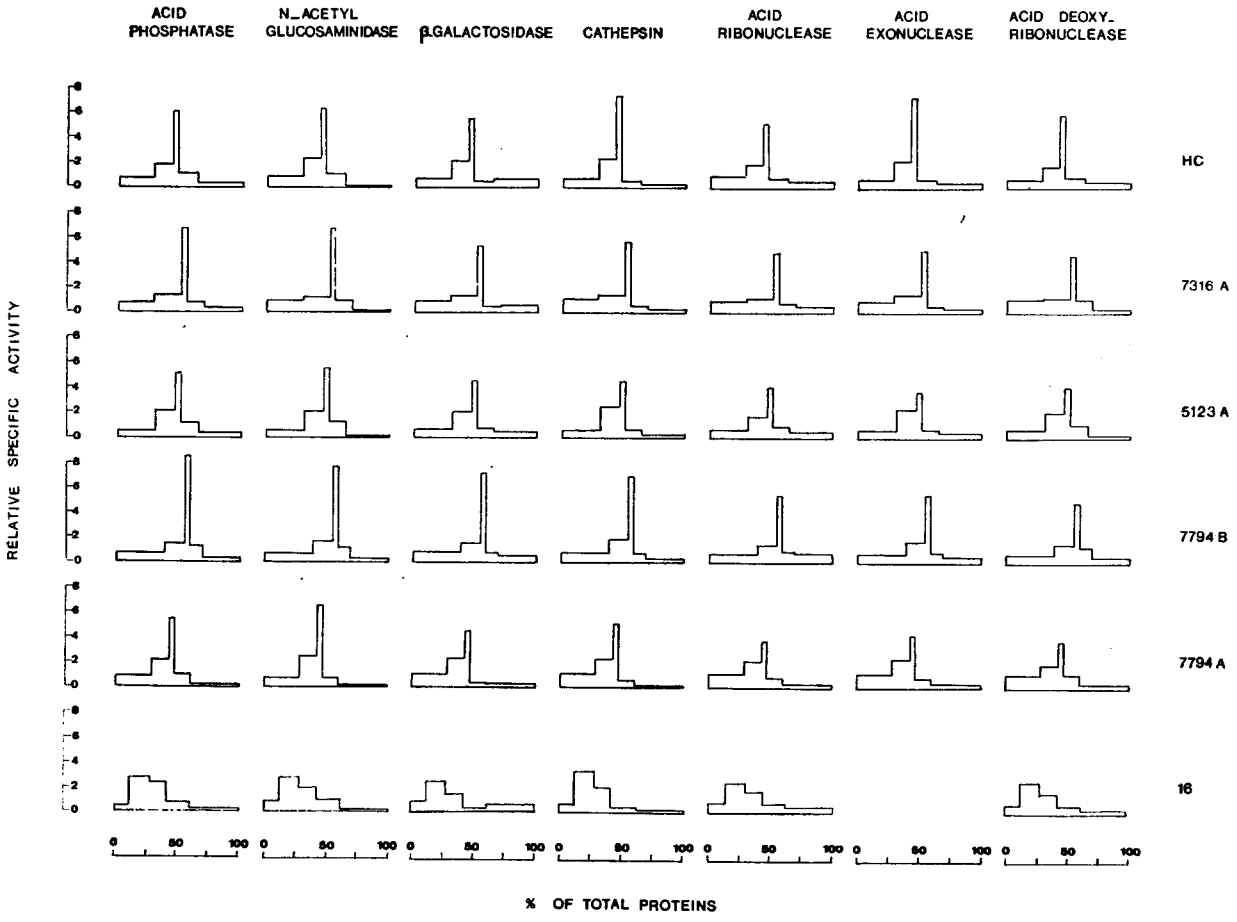


*Fig. 2. Distribution patterns of acid hydrolases.* Ordinate, *mean relative specific activity of fractions (percentage of total recovered activity/percentage of total proteins).* Abscissa, *relative protein content of fractions (cumulatively from left to right).*

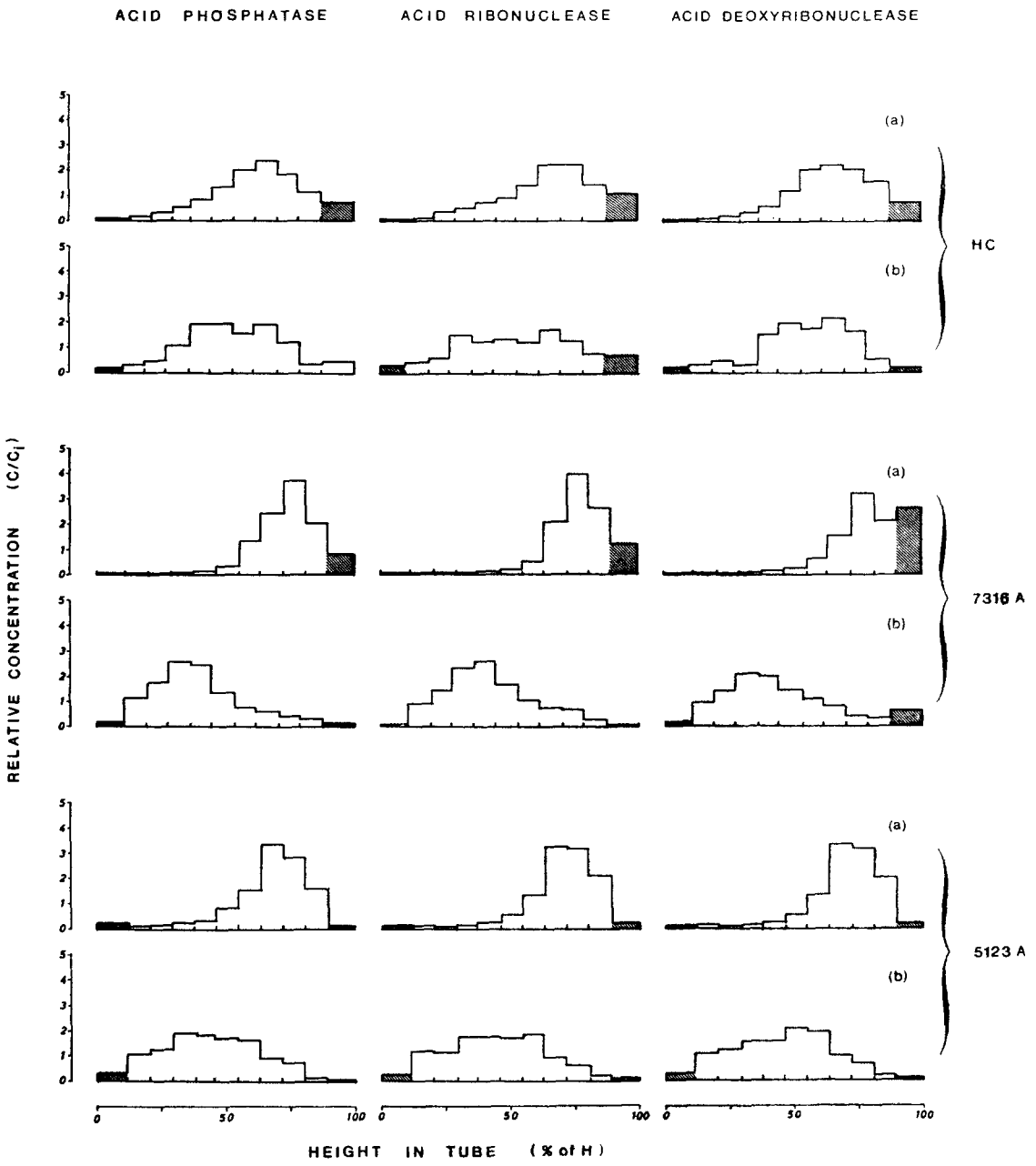


*Fig. 3. Density equilibration of particle-bound enzymes after centrifugation at* $1{\cdot}86\times10^7$ **g**.*min of a mitochondrial fraction of hepatoma HC, 7316A and 5123A, through a* 0·776 *to* 3·419 *molal sucrose gradient in water. The particles were initially layered on the top of the gradient.* Abscissa, *percentage of the height of the liquid column in the tube (H);* ordinate, *relative concentration, i.e. ratio of the observed activity (C) to that which would have been observed if the enzyme had been homogeneously distributed throughout the whole gradient (Ci). Filled blocks (▩) are used for the top and bottom subfractions to indicate that they include material falling beyond the limits of the gradient. (a) Normal rat; (b) rat injected intraperitoneally with* 200 *mg of Triton WR* 1339 *dissolved in saline and killed three days after injection.*

The number of individual determinations are given in parentheses.

*Sucrose gradient*

Figures 3 and 4 illustrate the distribution, after isopycnic centrifugation in a sucrose gradient, of acid phosphatase, acid ribonuclease and acid deoxyribonuclease taken as reference enzymes of lysosomes. No striking distribution differences are observed among hepatomas 7794A, 7794B, 5123A, 7316A and 16, the median equilibrium density being approximately 1·20 for the three hydrolases. Distribution of the enzymes is more heterogeneous in HC hepatomas and the median density is slightly lower (Table 2). Figures 3 and 4 also show the distributions obtained when the mitochondrial fraction is isolated from the

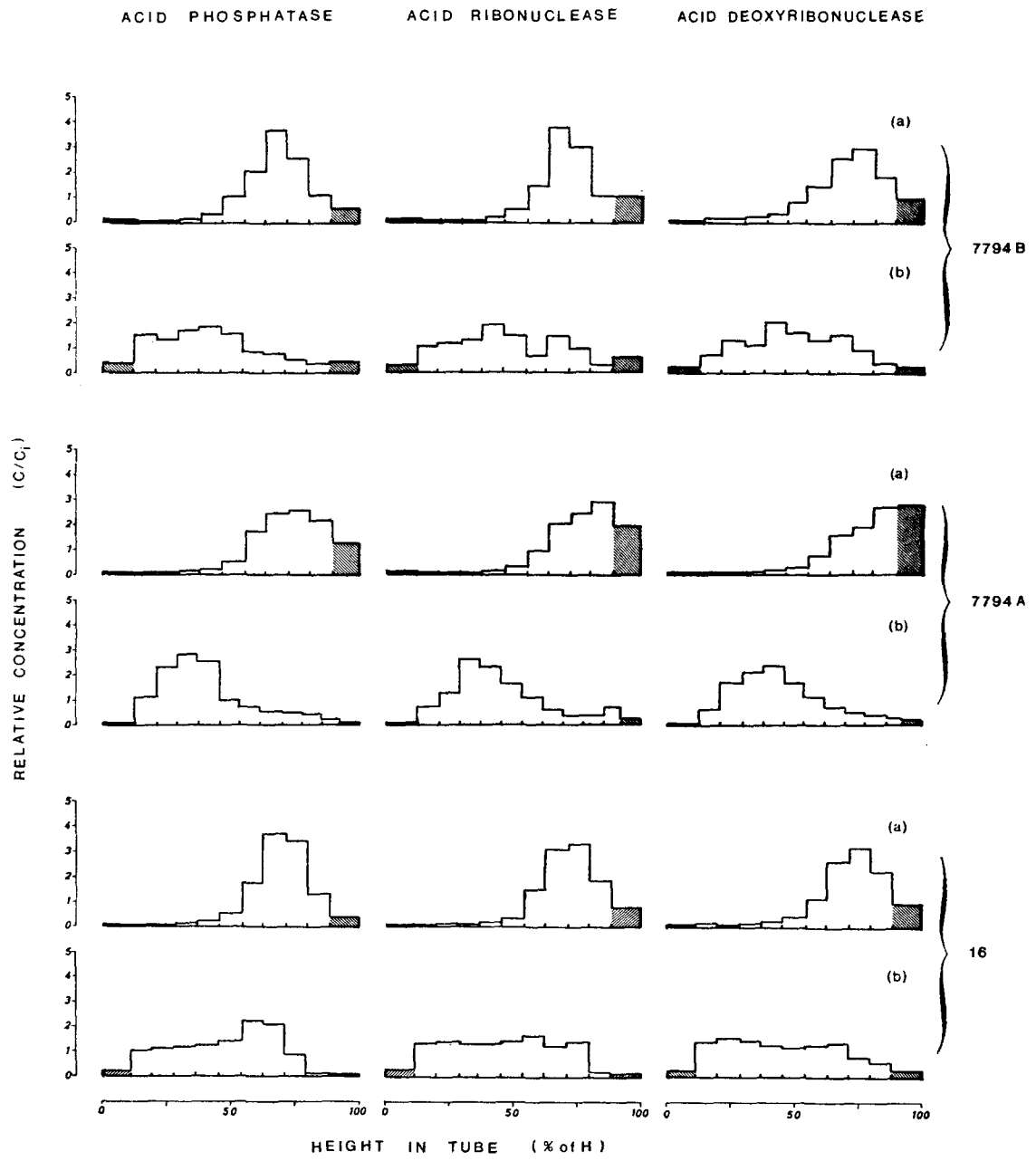


*Fig.* 4. *Density equilibration of particle-bound enzymes after centrifugation at* $1{\cdot}86\times10^7$ **g**.*min of a mitochondrial fraction of hepatoma* 7794*B*, 7794*A and* 16. *For explanation of the graph, see Fig.* 3.

tumor of a rat injected with Triton WR1339, a non-ionic detergent. The behaviour of the hepatoma lysosomes can be seen to be affected by pretreatment of the animal; the acid hydrolases are mostly recovered in a lower-density region, indicating a decrease in the equilibrium density of the enzyme-bearing granules (Table 2).

*Glycogen gradients*

The results are summarized in Fig. 4, where the median equilibrium densities observed in the different glycogen gradients have been plotted as a function of the sucrose concentration of the solution in which the polysaccharide was dissolved. (The method, by which the curves fitting these points was calculated, will be explained in the Discussion.) As shown in Fig. 5, the density of the granules increases with the sucrose concentration of the medium.

*Activation of lysosomal enzymes*

Lysosomal hydrolases are characterized by their structure-linked latency, which is due to the impermeability of the granule membrane to external substrates. Various chemical and physical agents are able to unmask the hydrolases by altering the lysosomal membrane [13].

*Table 2. Median equilibrium densities of acid hydrolases in sucrose—$H_2O$ gradients*

| | Median equilibrium density | |
|---|---|---|
| | Normal rat | Rat injected with Triton WR1339 |
| Hepatoma HC | | |
| Acid ribonuclease | 1·193 | 1·170 |
| Acid deoxyribonuclease | 1·188 | 1·173 |
| Acid phosphatase | 1·184 | 1·167 |
| Hepatoma 7794A | | |
| Acid ribonuclease | 1·214 | 1·134 |
| Adic deoxyribonuclease | 1·219 | 1·136 |
| Acid phosphatase | 1·204 | 1·124 |
| Hepatoma 7794B | | |
| Acid ribonuclease | 1·200 | 1·152 |
| Acid deoxyribonuclease | 1·203 | 1·156 |
| Acid phosphatase | 1·195 | 1·142 |
| Hepatoma 7316A | | |
| Acid ribonuclease | 1·208 | 1·142 |
| Acid deoxyribonuclease | 1·211 | 1·145 |
| Acid phosphatase | 1·205 | 1·136 |
| Hepatoma 5123A | | |
| Acid ribonuclease | 1·198 | 1·150 |
| Acid deoxyribonuclease | 1·198 | 1·154 |
| Acid phosphatase | 1·193 | 1·148 |
| Hepatoma 16 | | |
| Acid ribonuclease | 1·201 | 1·153 |
| Acid deoxyribonuclease | 1·204 | 1·150 |
| Acid phosphatase | 1·197 | 1·162 |

The effect of hypotonic treatment and incubation at pH 5 and 37°C in the presence of 0·25 M sucrose was tested on granule preparations isolated from hepatomas, using acid phosphatase as reference enzyme for lysosomes. The results are illustrated in Fig. 6. Free enzyme activity is increased by hypotonic treatment of the preparation. There are no important differences in the behaviour of granules isolated from hepatomas HC, 7316A, 16 and 5123A. A single experiment performed on hepatoma 7794A suggests that its lysosomes are comparatively more stable in hypotonic media. Free acid phosphatase activity is also increased by incubating the granules at pH 5 and 37°C for 80 min. The extent of activation varies from one hepatoma to the next; however the lysosomes of HC hepatoma appear to be significantly more resistant to such treatment.

## DISCUSSION

The distribution pattern of acid hydrolases following differential centrifugation resembles the distribution of the same enzymes in rat liver. However, it can be seen that, although there is a peak in the relative specific activity of the acid hydrolases in the light mitochondrial fraction *L*, the heavy mitochondrial fraction *M* contains the largest proportion of the activities of these enzymes. That is not the case in rat liver. Under these experimental conditions, the main factor governing the distribution of acid hydrolases is the size of the particle. It is therefore likely that the mean size of the lysosomes in the hepatomas we have investigated is larger than that of rat liver lysosomes.

The equilibrium density of the tumor lysosomes increases as a function of the medium sucrose concentration. Beaufay and Berthet [14] have shown that the behaviour of rat-liver hydrolases in sucrose and glycogen gradients may be explained by postulating that the lysosomes consist of an osmotic space, a space freely accessible to sucrose (sucrose space), and a hydrated matrix. As shown by de Duve *et al.* [15], the relationship between the density of such a particle and the density of the medium is as follows:

$$\rho_p = \rho_w \frac{\rho_a \alpha + (\rho_a + \rho_m \beta)_m}{\rho_a \alpha + \rho_w (1 + \beta)_m} \qquad \text{(a)}$$

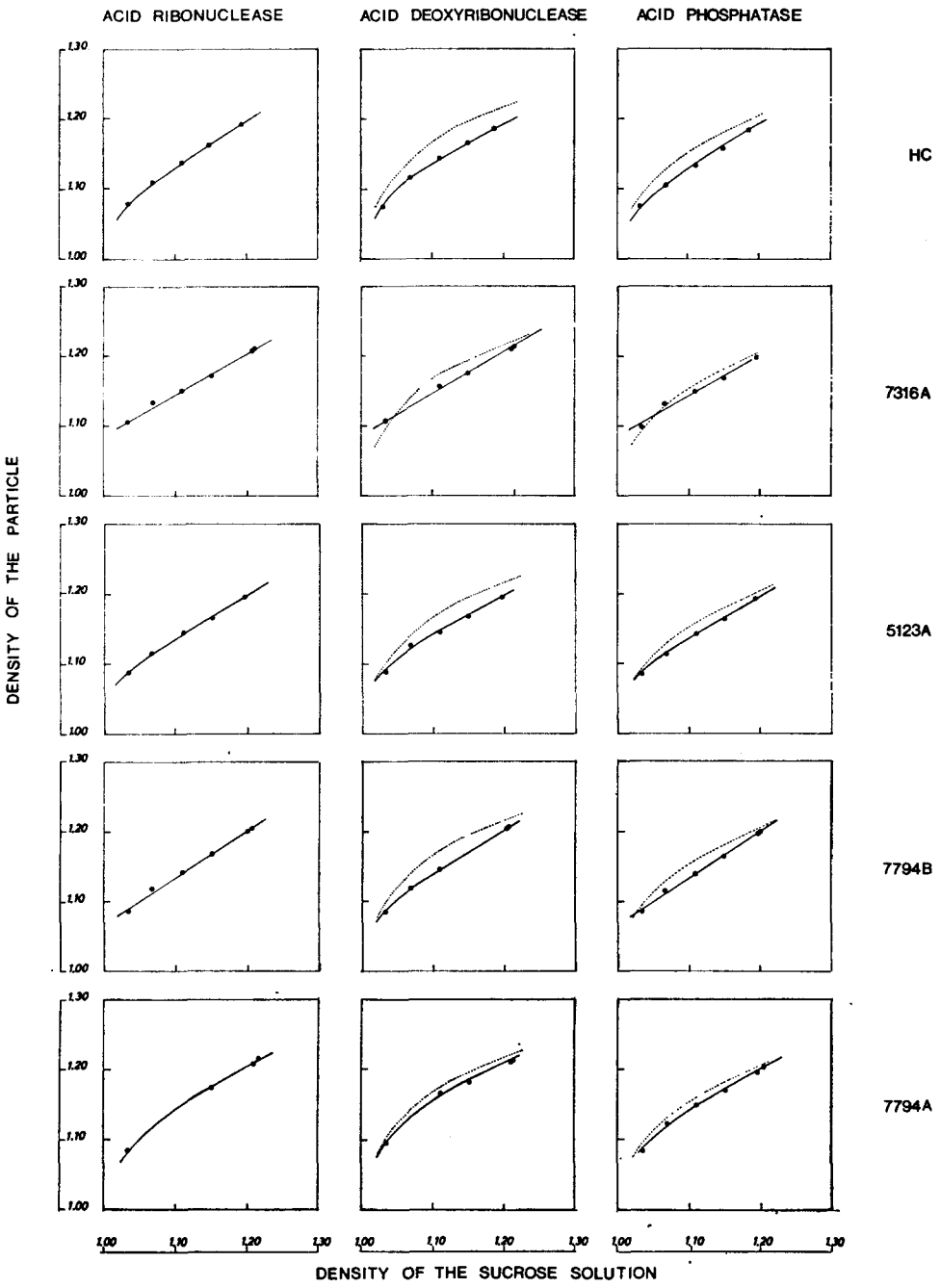


*Fig. 5. Median densities of the particles bearing hydrolases as a function of the density of the sucrose solution. The values were obtained by estimating the median equilibrium density in glycogen gradient and for the last point in sucrose gradient. The curves drawn fit equation (a) with the value of* $\alpha$, $\beta$ *and* $\rho_d$ *given in Table 3. In dotted line, the behaviour of rat liver enzymes according to Beaufay and Berthet* [14].

where $\rho_d$ is the density of the granule matrix which includes all the solid components of the particles and their hydratation water, $\rho_m$ is the density of the medium, $\rho_w$ the density of the solvent (pure water), $m$ the molality of the sucrose solution, $\alpha$ the particle relative content of osmotically active solutes, and $\beta$ the fraction of the particle that is freely accessible to sucrose.

The applicability of this model to hepatoma lysosomes was tested by calculating the most probable values for parameters $\alpha$, $\beta$ and $\rho_d$ of the equation (a) using the experimental points for each hepatoma. This was performed by the method of least squares with the aid of a computer. The calculated values are given in Table 3, together with the data reported by Beaufay and Berthet [14] for rat liver enzymes. The hepatoma granules are characterized by higher $\beta$ values and lower $\rho_d$ values. However, as pointed out by these authors, quantitative

*Table 3. Most probable values for the parameters* $\alpha$, $\beta$ *and* $\rho_d$ *of particles-bearing enzymes.* $\alpha$ *and* $\beta$ *are given respectively in milliosmole.g*$^{-1}$ *and cm*$^3$*.cm*$^{-3}$ *of hydrated matrix,* $\rho_d$ *in g.cm*$^{-3}$*. The values for the liver are those reported by Beaufay and Berthet* [14]

| | Hepatoma HC | Hepatoma 7794A | Hepatoma 7794B | Hepatoma 7316A | Hepatoma 5123A | Liver |
|---|---|---|---|---|---|---|
| Acid ribonuclease | | | | | | |
| $\alpha$ | 0·185 | 0·238 | 0·045 | 0 | 0·062 | |
| $\beta$ | 1·448 | 1·121 | 1·734 | 1·311 | 1·510 | |
| $\rho_d$ | 1·212 | 1·237 | 1·207 | 1·207 | 1·203 | |
| Acid deoxyribonuclease | | | | | | |
| $\alpha$ | 0·238 | 0·256 | 0·123 | 0·048 | 0·096 | 0·276 |
| $\beta$ | 0·912 | 0·545 | 1·380 | 1·145 | 1·194 | 0·340 |
| $\rho_d$ | 1·214 | 1·243 | 1·216 | 1·217 | 1·206 | 1·257 |
| Acid phosphatase | | | | | | |
| $\alpha$ | 0·220 | 0·177 | 0 | 0 | 0·074 | 0·102 |
| $\beta$ | 1·382 | 1·102 | 1·952 | 1·374 | 1·539 | 1·050 |
| $\rho_d$ | 1·206 | 1·220 | 1·195 | 1·202 | 1·200 | 1·226 |

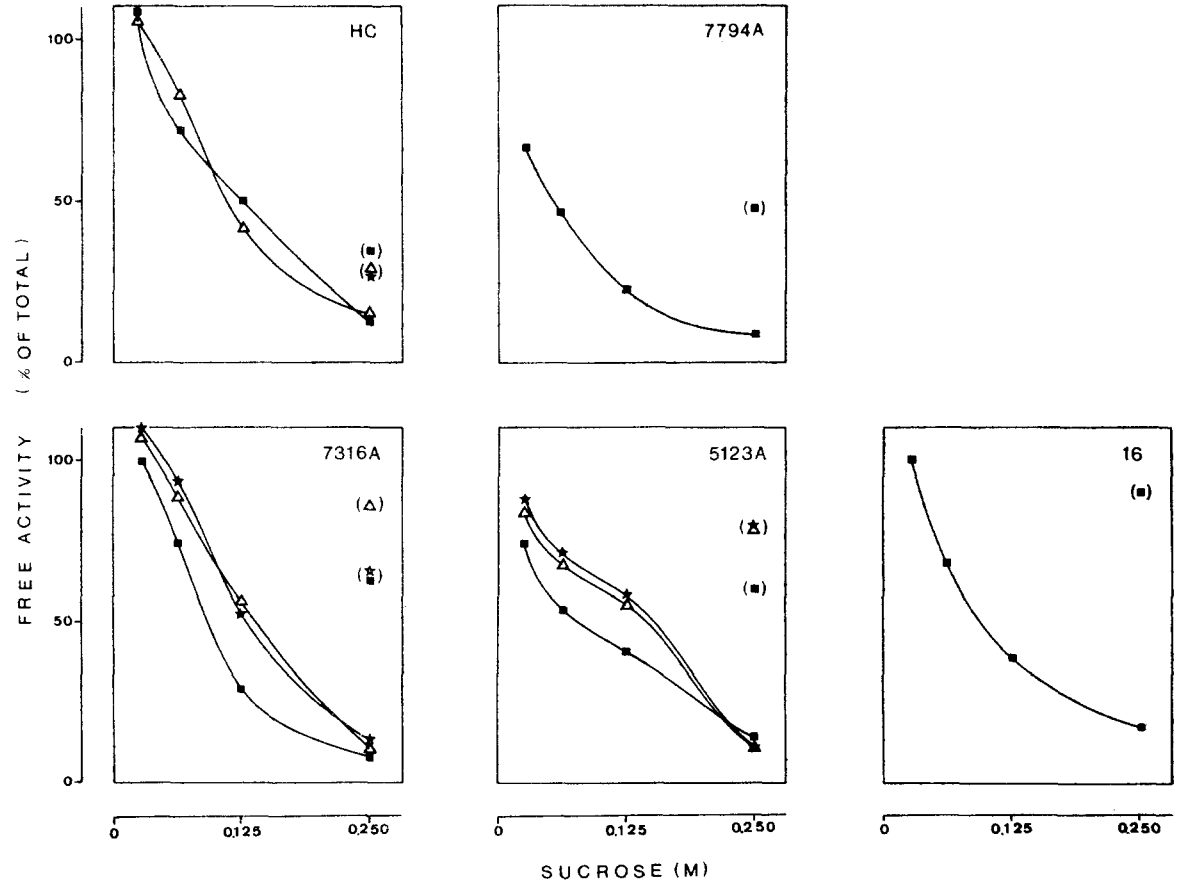


*Fig. 6. Activation of acid phosphatase by hypotonicity: samples of an hepatoma mitochondrial fraction prepared in* 0·25 *M sucrose were diluted with water to give the sucrose concentrations shown on the abscissa and kept* 10 *min at* 0°*C. After that, concentrated sucrose solution was added to restore isotonicity. Free activity was determined by assaying for* 10 *min in* 0·25 *M sucrose, total activity in* 0·1% *Triton X-*100 (9). *The symbols in brackets show the free activity measured after preincubation of the granules in* 0·25 *M sucrose at pH* 5 *and* 37°*C for* 50 *min. Each symbol represents a separate experiment.*

values concerning lysosomes such as those reported in Table 3 must be approached somewhat cautiously. The granule population is heterogeneous, as illustrated by the differences observed when acid phosphatase or deoxyribonuclease is taken as reference enzyme. Nevertheless, the differences reported here are observed in every tumor, which suggests that hepatoma lysosomes *in vitro* are relatively more permeable to sucrose than are liver granules.

The effect of injected Triton WR1339 on hepatoma lysosomes is particularly noteworthy. As has been shown [16], the injection of this non-ionic detergent markedly lowers the density of rat-liver lysosomes, a phenomenon which may be explained by the accumulation of the pinocytized detergent inside the organelles [16, 17]. This effect is of great interest in the study of lysosome function and also makes it possible to isolate highly purified lysosome preparations. Indeed, the density change in the granules leads them to band in a sucrose gradient in regions that are virtually free from other particles (mitochondria, peroxisomes, etc.) [16, 17]. In the present study, the accumulation of Triton WR1339 in hepatoma lysosomes was not studied in detail. Attention was primarily focused on the effect of the detergent on the equilibrium density of the lysosomes; in this respect, our results are quite demonstrative. It is to be pointed out that lysosomes of HC hepatoma exhibit the smallest decrease in equilibrium density. That suggests that lysosomes of this rapidly-growing tumor do not accumulate as much detergent as those of the other hepatomas. It would be interesting to enquire into the reason of that phenomenon. The effect of Triton WR1339 has already been observed on hepatoma HW lysosomes [18] and recently on lysosomes of Ehrlich ascite carcinoma cells [19].

The structure-linked latency of the lysosomal enzymes is one of their fundamental properties. In this study the phenomenon was observed for the acid phosphatase of hepatoma granules. The enzyme is unmasked to some extent when the granules are incubated at pH 5 and 37°C. However, only a slight activation is shown under the same conditions by the lysosomes of HC hepatoma, a rapidly growing tumor. A relatively high resistance to such treatment was observed by Horvat and Touster [20] for the lysosomes of Ehrlich ascites tumor cells, which grow rapidly. Wagner and Roth [21] report that lysosomal β-glucuronidase is only slightly unmasked by thermal treatment of granules originating from the rapidly growing Novikoff hepatoma whereas a clear-cut activation is observed when granules of the medium-slow growing Reuber hepatoma are tested. These findings suggest that there is a relationship between the growth of a hepatoma and the response of its lysosomes to thermal activation.

Hepatoma lysosomes are affected by the hypotonicity of the medium. This phenomenon seems paradoxical in the case of hepatoma 7316A lysosomes, which appear devoid or almost devoid of osmotic space as shown by gradient experiments. A plausible interpretation might be that the lysosomal membrane in this hepatoma becomes more permeable to sucrose during centrifugation in density gradients. This phenomenon might be due at least in part to an abnormal instability of the tumor lysosomes under the experimental conditions which prevail during gradient centrifugation experiments.

All of the seven hydrolases assayed in hepatomas were found to exhibit an activity more or less comparable to that found in rat-liver homogenate. This finding must be considered in the light of lysosomal function in tumors. Indeed, acid hydrolases are the agents which act in the lysosomal apparatus to degrade exogenous compounds taken up by the cell that reach the lysosomes, as well as endogenous substances during the process of autophagy [1].

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## SUMMARY

1. *The activities of seven acid hydrolases were measured in HC hepatoma and in Morris hepatomas* 7794*A*, 7794*B*, 5123*A and* 16. *In general the enzymes in the tumors exhibit a specific activity ranging between* 50 *and* 150% *of that found in rat liver tissue; however, the specific activities of acid phosphatase, N-acetylglucosaminidase and* ß*-galactosidase in hepatoma* 5123*A are far greater than those found in liver homogenates.*

2. *The intracellular distribution of these enzymes was established after differential centrifugation in* 0·25 *M sucrose. The distribution pattern resembles that of the same*

*enzymes in rat-liver homogenates. The enzymes are found mainly in the mitochondrial fractions and exhibit a peak of specific activity in the light mitochondrial fraction.*

3. *Total mitochondrial fractions of hepatomas were analyzed by isopycnic centrifugation in a sucrose gradient and in gradients composed of glycogen dissolved in aqueous sucrose of different molalities. A number of physical properties of lysosomes were deduced from the behaviour of acid hydrolases in these experiments. The hepatoma lysosomes seem to have a larger sucrose space than the liver organelles. Injection of Triton WR1339, a non-ionic detergent, causes a distinct decrease in the equilibrium density of hepatoma lysosomes in a sucrose gradient similar to that observed for rat-liver lysosomes.*

4. *Free activity of acid phosphatase, a reference enzyme for lysosomes, is increased by lowering the tonicity of the medium in which the granules are suspended and also by incubating the particles at pH 5 and 37°C in isotonic sucrose. HC hepatoma lysosomes appear to be significantly more resistant to this latter treatment than the granules of the other hepatomas.*

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# "Spontaneous" Neoplastic Transformation *in vitro*: the Ultrastructure of the Tissue Culture Cell

L. M. FRANKS and PATRICIA D. WILSON

*Department of Cellular Pathology, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, W.C.2, England*

## INTRODUCTION

A NUMBER of tissue-culture cell lines were established from different organs of young and old C3H and C57BL/Icrf/a$^t$ mice. Some of these produced tumours after subcutaneous inoculation into syngeneic mice [1]. The ultrastructure of 24 of these cell lines was studied, at different transfer generations, for demonstrable differences between tumour and non-tumour lines and between lines derived from different organs from young and old animals of the two mouse strains; the surface structure of one of these cell lines has already been described [2]. We have also tried to identify the origin of the cells which can be maintained in culture since this has bearing on the process of neoplastic change *in vitro*, whether spontaneous or induced by viruses or chemical carcinogens.

## MATERIALS AND METHOD

Tumour and non-tumour cell lines derived from kidney, lung, bladder, tongue, heart, spleen, prostate, peritoneum and spinal cord were examined at different transfer generations (Table 1). The origin of the cell lines and details of culture methods and transplantation techniques are described in an earlier paper [1]. Cells were removed from the culture vessels mechanically or by trypsinization, using the method by which the cells were routinely transferred (see [1] for details). The cell suspensions were fixed for 20 min in 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer pH 7·1 at room temperature, spun into a pellet and rinsed overnight in 0·1 M sodium cacodylate buffer at 4°C. Hand-chopped pellets were postfixed for 1 hr in Palade's fluid over ice, dehydrated in graded ethyl alcohols, stained with 5% uranyl acetate in absolute alcohol, and embedded in araldite, using epoxypropane as transitional solvent. Sections cut on a Sorvall MT-2 ultramicrotome were picked up on copper grids, stained with Reynold's lead citrate [3] and viewed in an Hitachi HS7S or Siemens Elmiskop 1 electron microscope. Suspensions of cells from trypsinized tissues which had not been cultured were similarly treated for electron microscopy Two of these suspensions were from kidneys from 3- and 5-day-old mice and 2 from 32-month-old mice.

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## RESULTS

In all the cell lines examined 2 main types of cell were identified. Type 1 cells (Figs. 1 and 2) usually had a large rounded or bean-shaped nucleus with a very thin layer of condensed chromatin against the nuclear membrane. Small clumps of chromatin were associated with a prominent usually single nucleolus. The cytoplasm contained some rough endoplasmic reticulum, many free ribosomes, often

*Table 1. Ultrastructural features of tumour and non-tumour cell lines*

| Expt. | No. | organ | Mouse strain | Age | Transfer generations examined | Glycogen | Virus† | | Collagen | Specialized contacts | | | Intra-nuclear bodies |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Intra-cellular | Extra-cellular | | 1–1 | 1–2 | 2–2 | |
| CBM | 15 | Kidney | C57 | 3 days | 2, 6, 13 | − | − | − | − | − | − | + | + |
| CBM | 17 | Kidney* | C57 | 3 days | 5, 11, 21, 28, 40, 43, 64 | + | + | − | + | − | + | − | + |
| CBM | 18N | Kidney* | C3H | 3 days | 7, 14, 24, 29, 34 | + | + | + | ± | + | − | + | + |
| CBM | 18S | Kidney | C3H | 3 days | 6, 13, 20 | − | − | − | − | − | − | − | − |
| CBM | 19 | Lung* | C57 | 3 days | 9, 15, 23, 40 | + | + | + | − | − | − | + | + |
| CBM | 23 | Lung | C3H | 3 days | 7, 15, 19 | − | + | + | − | − | + | + | − |
| CBM | 25 | Kidney* | C57 | 3 days | 7, 12 | + | − | − | − | − | − | − | − |
| CBM | 26 | Kidney* | C57 | 3 days | 4, 18 | + | − | − | − | − | − | + | + |
| CBM | 29 | Heart | C3H | 3 days | 5, 11, 16 | − | + | + | + | − | − | + | + |
| | | Spleen | | | 2 | − | − | + | + | − | − | + | − |
| CEM | 1 | (Embryo) | C57 | 18 days embryo | 6, 9, 14, 18 | − | − | − | + | − | − | + | − |
| COM | 1 | Heart* | C57 | 29 months | 4 | − | − | − | − | + | + | + | + |
| COM | 2 | Lung* | C57 | 28 months | 3, 9 | − | − | − | + | + | + | + | − |
| COM | 3 | Kidney | C3H | 30 months | 2, 6, 11 | − | + | + | + | + | + | + | + |
| | | Lung | | | 9, 13 | − | + | + | − | − | − | + | − |
| COM | 4 | Bladder* | C57 | 34 months | 5 | + | − | − | − | − | − | + | + |
| | | Kidney* | | | 5 | − | − | − | + | − | − | + | − |
| | | Prostate | | | 4, 14 | − | − | − | − | − | − | − | + |
| COM | 5 | Tongue* | C57 | 28 months | 2, 15, 18, 26, 28 | + | + | + | + | − | + | + | + |
| | | Peritoneum | | | 3 | − | ⊕ | ⊕ | + | − | − | − | − |
| | | Kidney | | | 5 | − | + | + | − | − | − | − | + |
| COM | 6 | Peritoneum | C3H | 29 months | 4 | − | + | + | − | − | − | + | − |
| | | Spleen | | | 6 | − | + | + | − | − | − | + | − |
| | | Spinal Cord* | | | 1, 3, 5, 15 | − | + | + | + | + | + | + | + |

* Tumour-producing lines.
† "C particles".
⊕ Polyoma-type particles also present.

in rosettes, a recognizable Golgi zone and relatively few lysosomes, autophagic vacuoles and mitochondria. Cell processes were short and few in number. Microvilli were very scarce.

Type 2 cells (Figs. 3–5) had a more convoluted nucleus in which the peripheral chromatin layer was thicker and small clumps of chromatin were scattered throughout the nuclear matrix and associated with prominent nucleoli. The difference in nuclear pattern was the most distinctive feature between the 2 cell types. The cytoplasm usually contained a large Golgi zone, many free ribosomes and, particularly in the peripheral zone, many lysosomes, autophagic vacuoles and mitochondria. The rough endoplasmic reticulum was less abundant than in the type 1 cells but the cisternae were often distended with finely granular material. The cell surface was irregular with many thin convoluted processes. Sheet-like cytoplasmic fringes often extended for a considerable distance from the cells. In suspensions prepared by scraping they were usually oriented along a layer of extracellular fibrillar material (Fig. 3) and often interdigitated with similar processes from other cells. Microvilli were present in some cells but absent from others. In suspensions prepared by trypsinization many of the cells were rounded but others still had many long processes (Fig. 4). A single cilium projecting into a small vacuole (Fig. 5) was found in type 2 cells in 6 cultures — 3 from young mice and 3 from old. Centrioles were seen only occasionally.

Although most cells could be easily classified as type 1 or 2, there were some atypical cells in most cultures. Most of these had the cytoplasmic characters of type 1 cells but the nuclei were much more convoluted, although the chromatin pattern was similar to that normally found in type 1 cells (Fig. 6). Other cells also had more cytoplasmic organelles than the typical type 1 cell suggesting that there may have been a transition from type 1 to type 2 cells. Occasional giant cells, sometimes multinucleate but usually with a single nucleus, were also found. Only their size distinguished them from other type 1 and 2 cells.

*Features common to all cell types*

All cell types had a number of features in common. Nuclear bodies were seen in many cells (Table 1) similar to those described by Bouteille *et al.* [4] (types 1–4 in their classification). Occasional nuclei contained lamellar structures resembling myelin figures. Bundles of intranuclear fibrils about 40–50Å in diameter were found in one cell line. Intranuclear cytoplasmic inclusions were common. Pinocytosis was frequent and the cytoplasm contained many large and small smooth membrane-bounded vesicles. Phagocytosis of cell debris or sometimes of whole cells was also found frequently but was more common in type 2 cells.

Glycogen deposits were found in most transfer generations of some tumour-producing cell lines from C57 and C3H mice (Table 1) as aggregates of single, roughly isodiametric beta particles 150–300Å in diameter. Long "dense" mitochondria were often present beside the glycogen deposits (Fig. 6).

The appearance of the mitochondria varied considerably. Normal mitochondria with comparatively light matrix were found in cells of most cultures. In some cases these mitochondria were greatly swollen (Fig. 2), or contained very short cristae. "Dense" mitochondria were also abundant in all cultures. These were characterized by a very dense matrix and distorted cristae (Fig. 7), sometimes in a tubular pattern. Both "normal" and "dense" mitochondria were found within the same cell.

Intracytoplasmic filaments were present in most cells (Figs. 8 and 11), usually randomly distributed but sometimes arranged in broad bundles beneath the plasma membrane or next to and parallel with the nuclear membrane. Most individual filaments were about 80 Å in diameter and in favourable sections could be seen to have a substructure resembling that of the actin-like filaments described in many other cells (5 for review). Other filaments about 35–40 Å were also present in some cells. Fusiform dense bodies resembling those seen in smooth muscle cells were seen very rarely. The filaments were most abundant in type 2 cells. A complex system of microtubules was also present. There was no regular arrangement of the microtubules although they sometimes ran along the long axis of cytoplasmic projections.

Specialized cell contacts (Figs. 8–11) between type 2 cells (Figs. 10 and 11) were found in most cultures, but contacts between type 1 and 2 cells were also seen (Table 1). The majority resembled the intermediate junctions (zonula adherens) (Fig. 9) of epithelial cells [6] but a few atypical tight junctions (zonular occludens) (Fig. 11) were also found. Condensations of amorphous material (Fig. 12) on the inner aspect of the plasma membrane, resembling, but smaller than, the attachment bodies of smooth muscle [7], were not infrequent and

were often associated with extracellular material resembling basal lamina (Fig. 12). This material was associated with many type 2 cells and some type 1 cells in all cultures, but in no case was a cell completely surrounded. The dense component, varying in width but usually about 300–400 Å, had a finely filamentous substructure and was separated from the cell by an electron lucent zone about 150–200 Å wide. Other extracellular material was found in all cultures. The amount present was not related to the age of the culture but appeared increased in the presence of type 2 cells. The material was made up of 2 components, an amorphous material and fine fibrils about 100 Å in diameter. On cross section these had a tubular appearance with an electron lucent core and a denser outer rim (Fig. 13). Also small amounts of banded collagen with a repeat period of circa 530 Å (Fig 14) were found in some cultures. This periodicity is normal for collagen in our embedded material.

Virus-like particles were present in many cell lines (Table 1). Most were C particles [8] and were extracellular, in cytoplasmic vacuoles or developing from plasma or vacuole membrane (Fig. 15). Occasional intracytoplasmic A particles were seen. In one series of cultures from an old mouse (COM 5 peritonium) intranuclear, cytoplasmic and extracellular particles of polyoma type [9] were found (Figs. 16 and 17).

Except for the presence of glycogen in some tumour lines, there were no morphological differences or differences in the proportion of the 2 cell types between tumour and non-tumour lines from young and old mice, or between different transfer generations or between lines derived from different mouse strains (Table 1).

*Non-cultured cell suspensions*

These cell suspensions were prepared in exactly the same way as cell suspensions which were used as the starting inocula for tissue cultures. The kidney suspensions invariably contained many free cells and fragments or renal tubules and glomeruli. Cells resembling type 2 cells remained attached to the tubules and it could be clearly seen that these cells lay outside the tubular basement membrane, in a site where the peri-tubular capillaries are found. Similar cells were also present closely associated with the basal lamina of glomerular capillaries (Figs. 18 and 19). These partially detached cells have many processes resembling those seen in the cells *in vitro*. The free cell suspensions from these preparations contained cells almost identical in structure with type 1 and type 2 cells seen in the cultures but type 1 cells were relatively uncommon (Fig. 20).

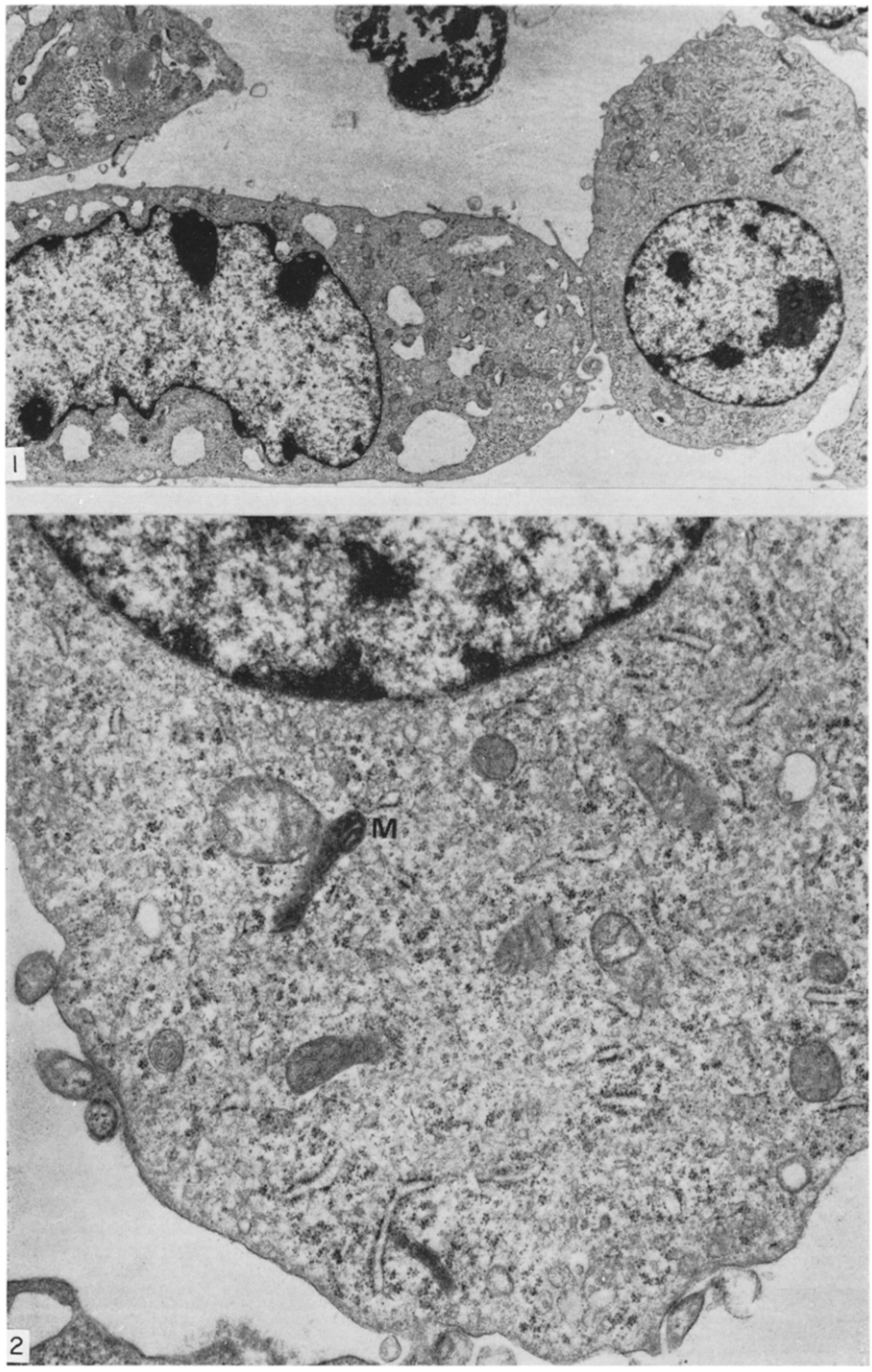
## DISCUSSION

Although "spontaneous" transformation *in vitro* has been studied for many years there have been few electron microscopic investigations of the cells involved, except for the recent report by Cornell [10], who concluded that the cells were probably fibroblasts. Although the cells present in our cultures are similar in many respects to those described by Cornell [10] we have recognized two distinct cell types in all cultures, whatever the organ of origin. The presence of specialized cell contacts, the formation of material resembling basal lamina and the fact that the majority of the extracellular material formed is not collagen suggests that the cells are not fibroblasts. The possible nature of these cells is discussed later. We have been unable to demonstrate any significant differences between tumour and non-tumour cell lines, except for the presence of cytoplasmic glycogen deposits in the former.

*Structural changes in the cell lines*

The cytoplasmic accumulation of glycogen in the tumour lines suggests a disturbance in glucose metabolism. Similar glycogen accumulation has been found in some hepatic adenomas [11] and Sanford and her colleagues [12, 13, 14] have found biochemical evidence of altered glycolytic activity in some transformed cells. Structural changes suggesting other metabolic disturbances were found in all of the cell lines and included: intranuclear bodies [15], nuclear fibrils [16], dense mitochondria [17, 18] and large mitochondria of normal density.

The presence of virus particles did not seem to be associated with malignancy. One of the strains of mouse used (C3H) carries the Bittner agent while the other does not, but this does not appear to influence the occurrence of neoplastic transformation. Virus particles (C type) seen on electron microscopy are more common in the non-tumour lines (9/13 examined) than in the tumour lines (5/11). Electron microscopy is an inefficient method for establishing the presence of virus and the true incidence is probably higher. Hall *et al.* [19] who used electron microscopy and complement fixation techniques also found evidence of the presence of C particles in many mouse cell lines. It is possible that some of the tumour cell lines may be free from virus although this has not been established with




*Figs.* 1 *and* 2. *Type* 1 *cells from CBM* 17/64/*kidney* (*T*) *showing round or bean-shaped nucleus with thin peripheral layer of condensed chromatin. The cytoplasm contains some rough endoplasmic reticulum, many free ribosomes and relatively few organelles except for mitochondria. One mitochondrion* (*M*) *which has a very dense matrix lies next to a swollen mitochondrion. Fig.* 1 × 5000, *Fig.* 2 × 25,000.

*The cell cultures are described as follows: prefix CBM* = 3–20-*day mouse, COM* = 28–34-*month mouse. Expt. No.; transfer generation No.; tissue of origin. Suffix* (*T*) = *tumour line,* (*NT*) = *non-tumour line, e.g. CBM* 17/64/*kidney* (*T*). *See Table for full details.*

*Figs.* 1, 2, 4, 6, 8–11, 14, 18–20 *are from trypsinized cell suspensions.*

*Figs.* 3, 5, 7, 12, 13, 15–17 *are from mechanically prepared suspensions.*

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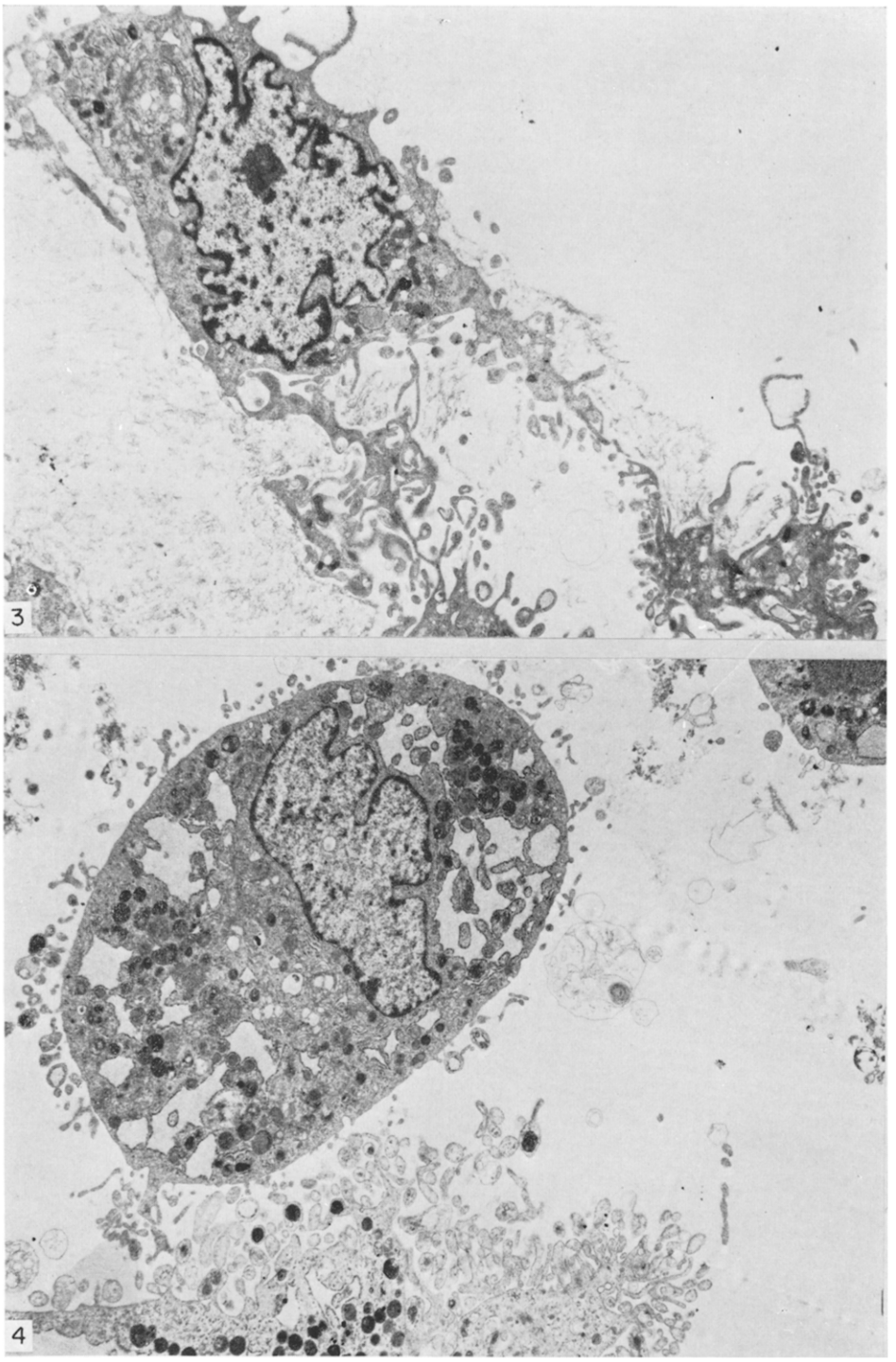


*Fig.* 3. *Type* 2 *cell from CBM* 29/16/*heart* (*NT*). *The cell suspension was prepared mechanically. The nucleus is convoluted and has a thick peripheral layer of chromatin. Convoluted processes extending along a layer of extracellular material are present at the lower pole of the cell and interdigitate with similar processes from other cells.* ×5000.

*Fig.* 4. *Type* 2 *cells from CBM* 26/8/*kidney* (*T*). *The cell suspension was prepared by trypsinization. The upper cell is rounded. The nucleus is convoluted and the cytoplasm contains dilated cisternae of rough endoplasmic reticulum and many cytoplasmic inclusions. The convoluted processes of another cell can be seen below.* ×5000.

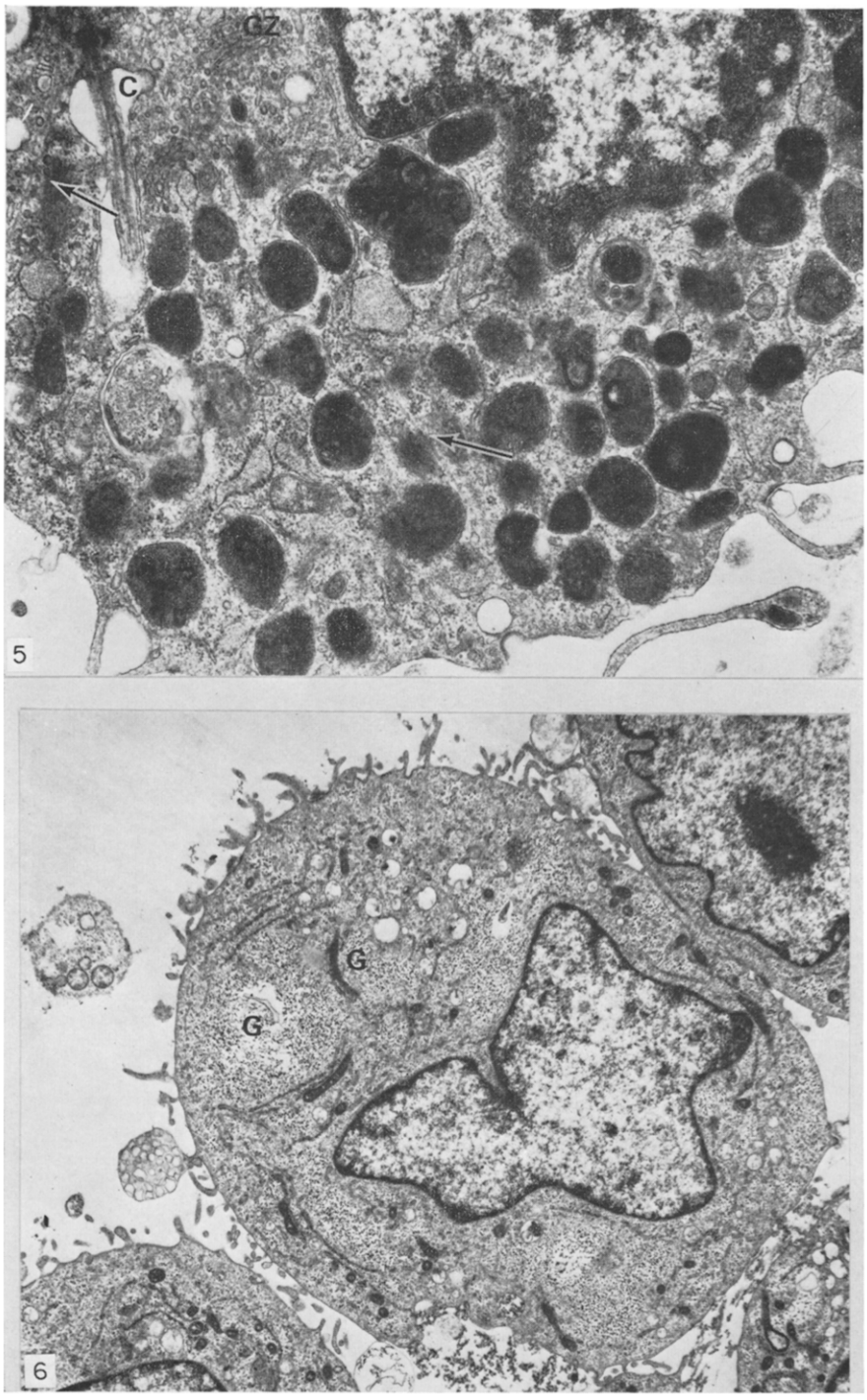


*Fig.* 5. *A type* 2 *cell from CBM* 25/7/*kidney* (*T*) *selected to show nuclear pattern and many cytoplasmic inclusions, Golgi zone* (*GZ*) *microtubules* (←————) *and a single cilium* (*C*). ×25,000.

*Fig.* 6. *Cell from CBM* 17/40/*kidney* (*T*) *showing glycogen deposits* (*G*) *many with dense mitochondria at their margins.* ×10,000.

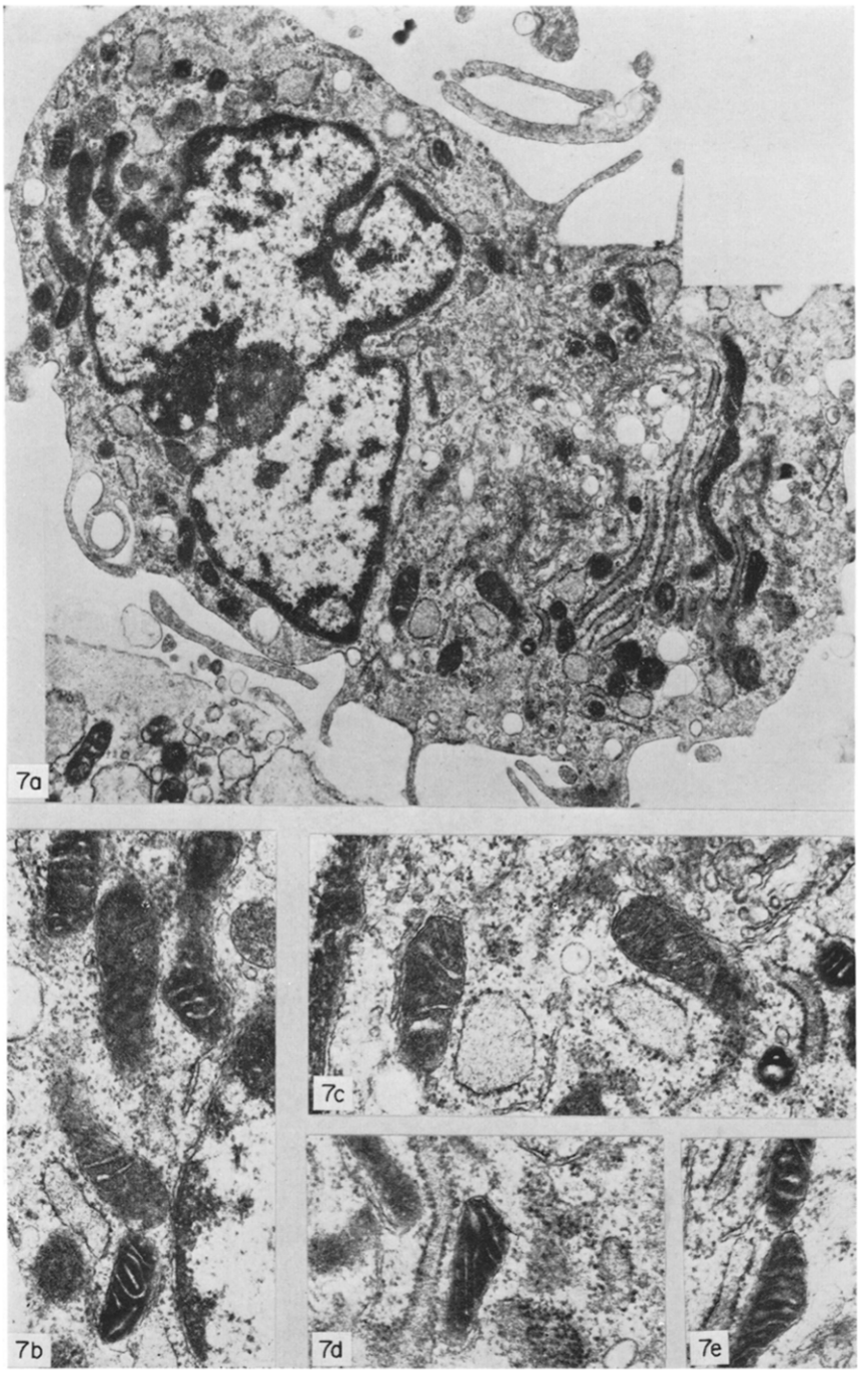


*Fig. 7a. A type 2 cell from CBM 23/7/lung (NT) showing great variation in mitochondrial pattern and matrix density in the same cell.* ×15,000.

*Fig. 7b–e. Higher magnification of mitochondria from cell in Fig. 7a, showing detail.* ×40,000.

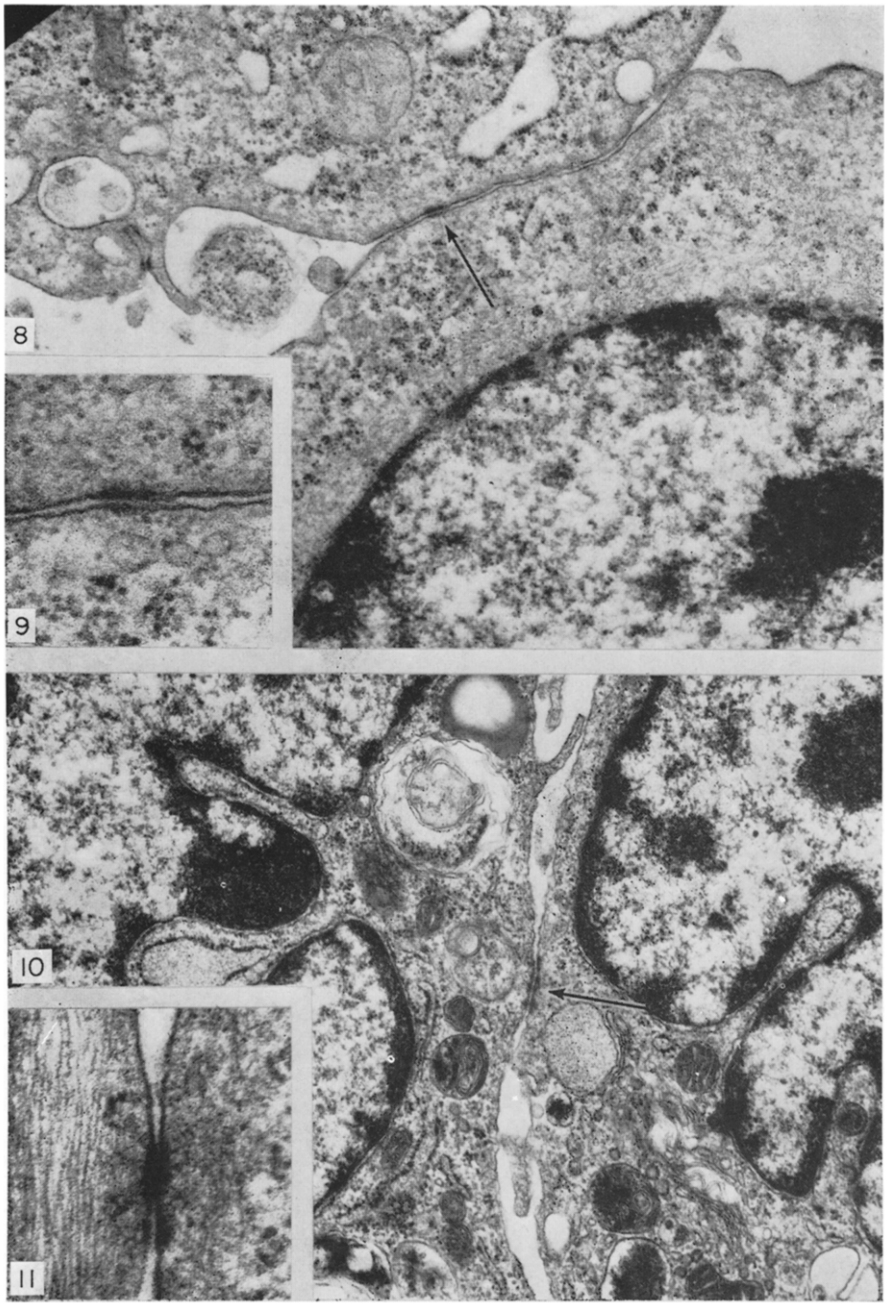


*Fig.* 8. *An intermediate junction between* 2 *type* 1 *cells from CBM* 17/64/*kidney* (*T*). ×25,000.
*Fig.* 9. *The junction shown in Fig.* 8. ×90,000.
*Fig.* 10. *An intermediate junction between* 2 *type* 2 *cells from CBM* 15/6/*kidney* (*NT*). ×25,000.
*Fig.* 11. *An atypical tight junction between* 2 *type* 2 *cells from CBM* 17/43/*kidney* (*T*). *There is no intercellular space in this region. A group of cytoplasmic fibrils can be seen in the cell on the left.* ×90,000.

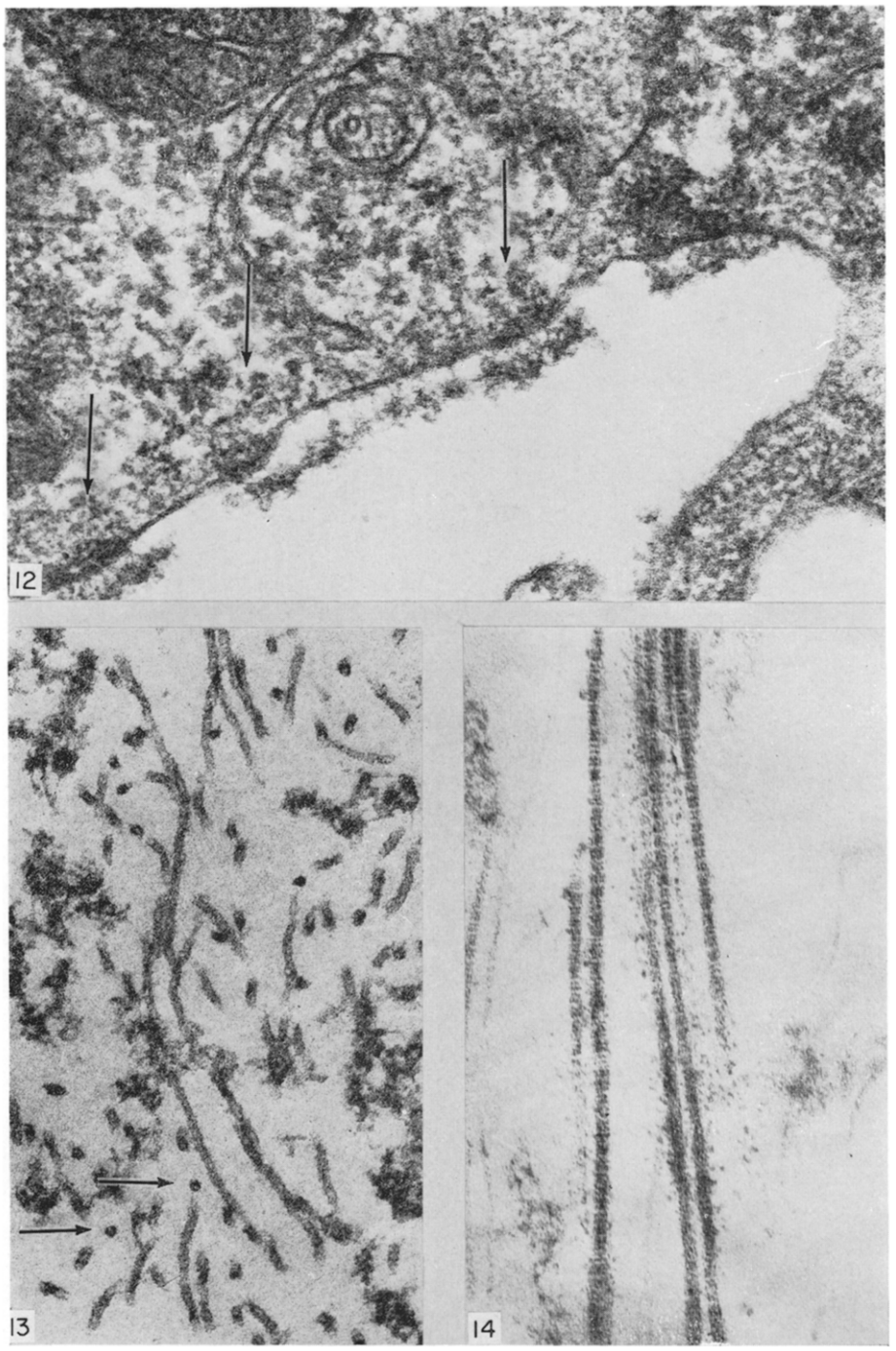


*Fig.* 12. *A cell from COM* 4/5/*bladder* (*T*) *showing small areas of increased density on the plasma membrane* (←) *and a layer of extracellular material resembling basal lamina.* × 120,000.

*Fig.* 13. *Fibrillar extracellular material from same grid as Fig.* 13 *showing longitudinal and transverse sections of fibrils. The electron lucent core and denser outer rim can be seen in transverse sections* (→). × 120,000.

*Fig.* 14. *Banded collagen fibrils from CBM* 17/21/*kidney* (*T*). × 120,000.

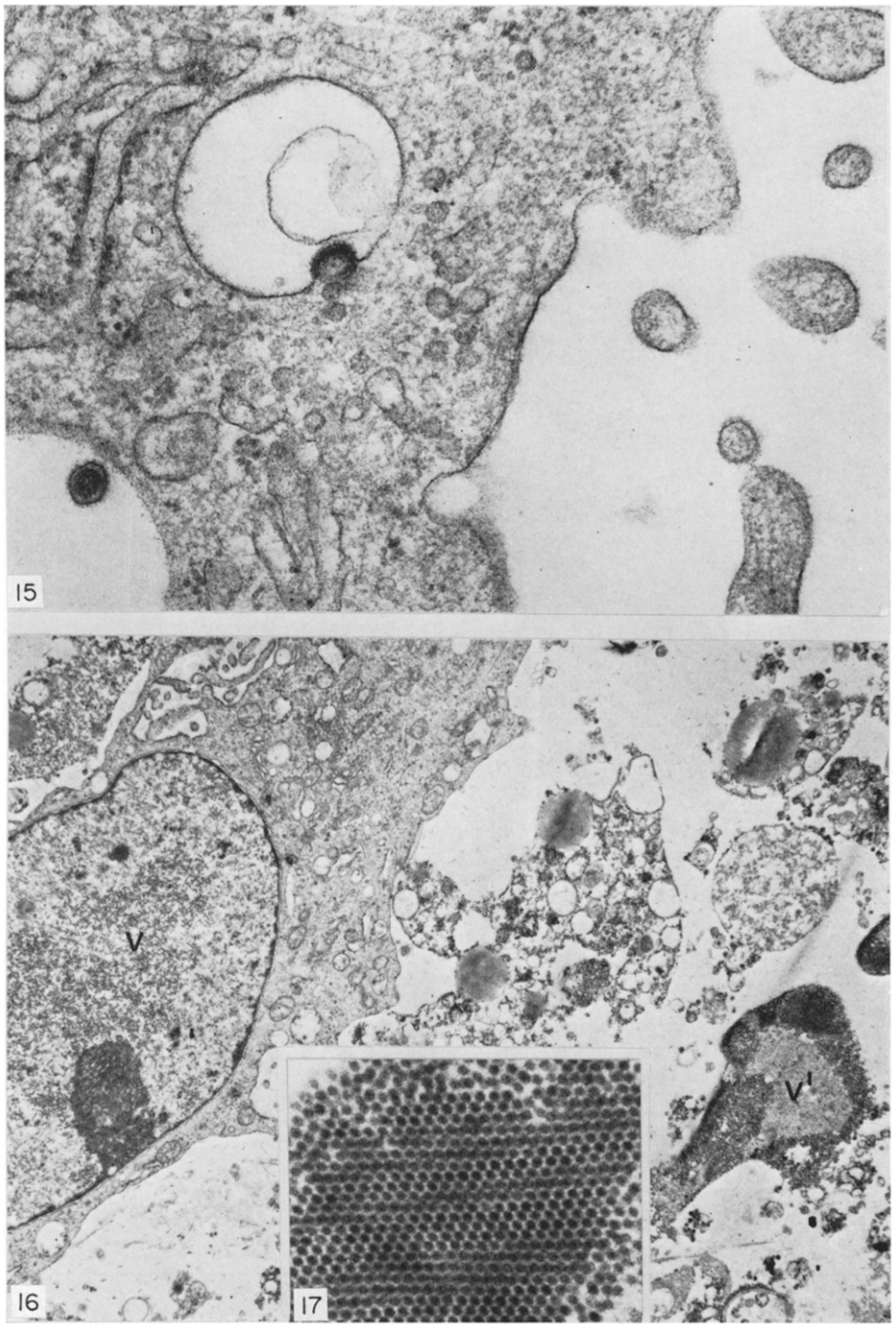


*Fig.* 15. *Mature and developing C particles in intracytoplasmic vacuoles in a type* 1 *cell from CBM* 23/15/ *lung.* (*NT*). ×90,000.

*Fig.* 16. *Intranuclear virus particles* (*V*) *of polyoma type in a type* 1 *cell from COM* 5/3/*peritoneum* (*NT*). *There is also an extracellular group of similar particles* (*V'*). ×9000.

*Fig.* 17. *A higher magnification of the extracellular particles in Fig.* 16, *showing crystalline array.* ×60,000.

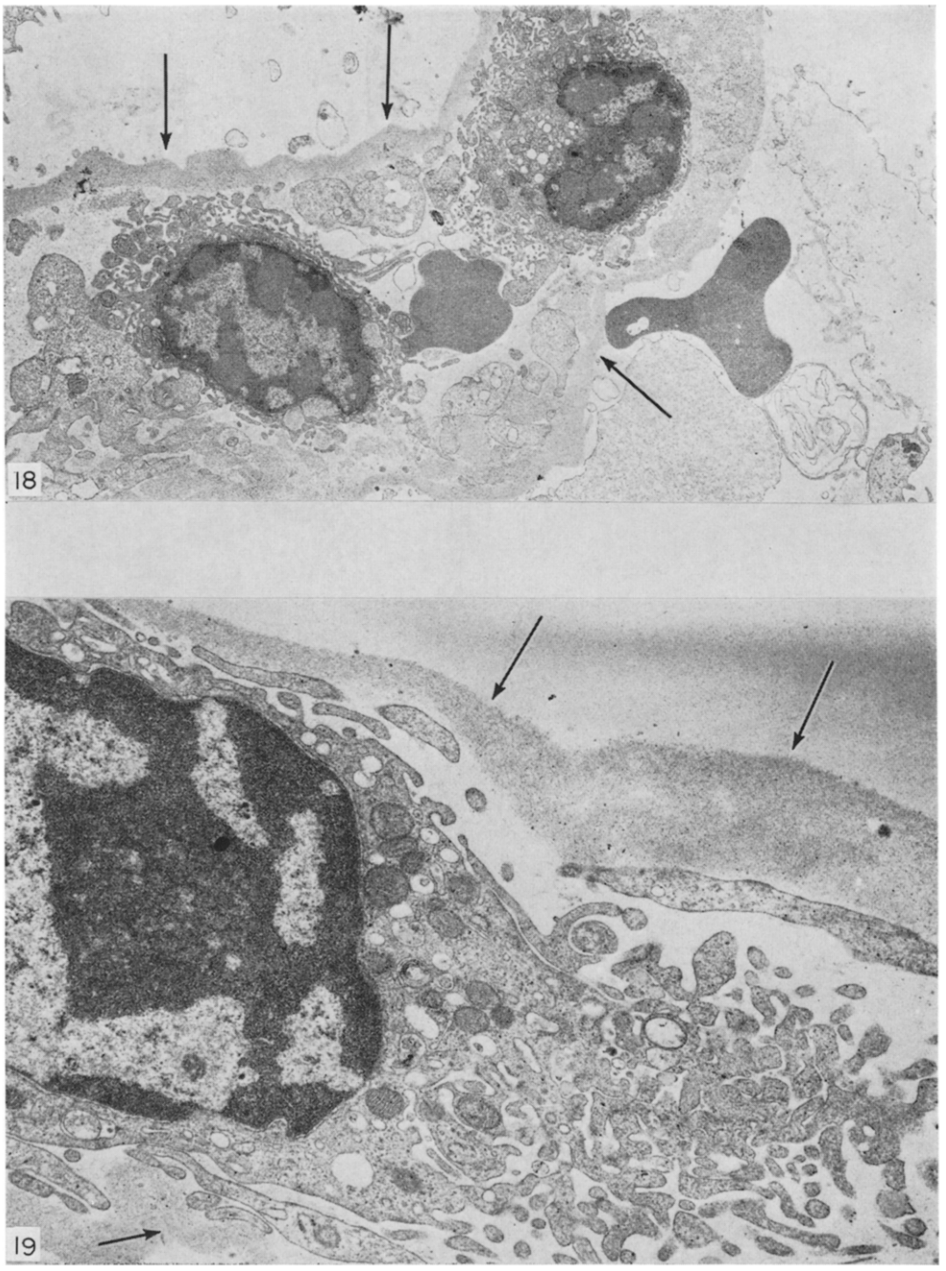


*Fig.* 18. *From a trypsinized suspension of a* 32*-month old mouse kidney. A low-power picture showing* 2 *cells with many convoluted processes closely associated with the thickened basal lamina* ( ↑ ↑ ) *of a glomerular capillary.* ×3500.

***Fig.*** 19. *A similar cell from the same preparation. There is basal lamina* ( ↑ ↑ ) *on either side of the cell. The nuclear pattern and cytoplasmic processes are similar to those of type* 2 *cells. Note the resemblance to the cell processes in Fig.* 4. ×25,000.

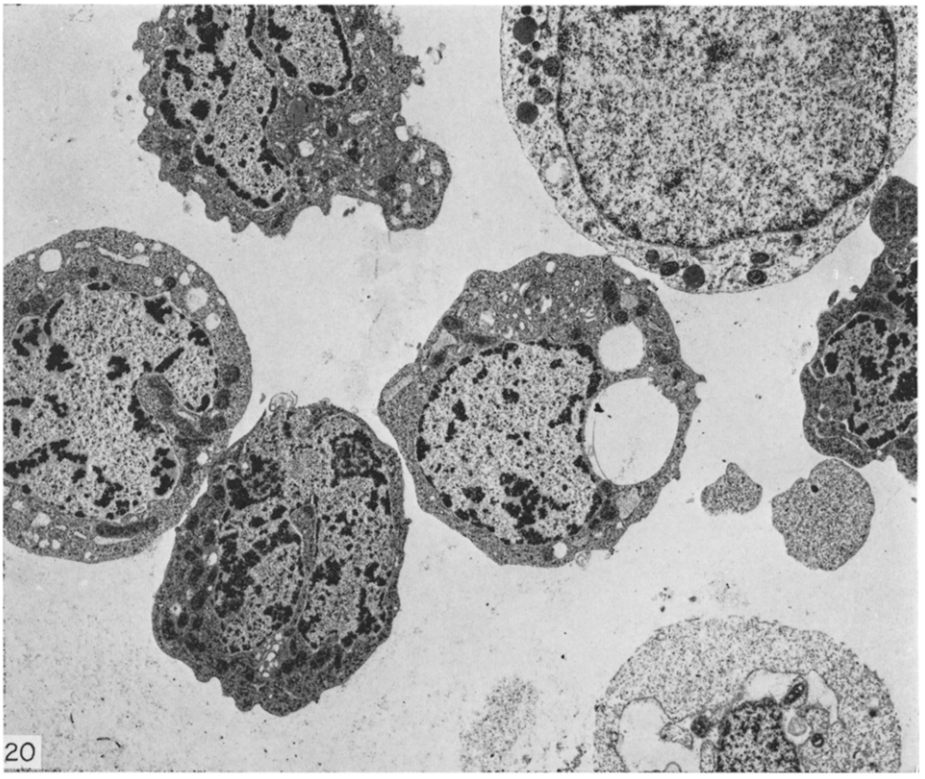


*Fig.* 20. *A group of cells from a trypsinized* 3*-day old mouse kidney. One cell (top right) resembles a type* 1 *cell and the others are similar to type* 2 *cells.* $\times$ 5000.

certainty. We have not been able to demonstrate any morphological differences (except for glycogen deposits) between tumour and non-tumour lines from young and old mice or between different transfer generations. More detailed functional and biochemical studies of these cells during the process of neoplastic transformation are in progress.

*The nature of the tissue culture cells*

The nature of cells which can be maintained *in vitro* has been a topic for discussion since tissue cultures were first initiated [see Refs. 20 and 21 for full discussion]. Although at least 6 different cell types can be recognized in primary explant cultures "one curious feature of pure cell strains is that at any one time there often, if not always, appear to be 2 morphological classes of cells in them, the spherical or somewhat spindle-shaped type on the one hand and the extended, flattened type on the other" [21]. The finding in our experiments of 2 main cell types in cultures derived from a wide range of organs — kidney, lung, heart, spleen, bladder, prostate, tongue, spinal cord and peritoneum — suggest that the cells may originate from a tissue common to all.

Alternatively, the appearance of the 2 cell types may be a direct consequence of the tissue culture environment and may represent a structural modulation rather than a specific selection of 2 cell types from the original mixed cell starting inoculum. However, this seems unlikely since some cells similar in morphology to the tissue culture cells can be seen in non-cultured cell suspensions. The possibility that one cell type may be derived from the other cannot be excluded, particularly as type 1 cells seem to be less common in the original suspensions. Experiments to test these possibilities are in progress. The ultrastructure of the cells in our cultures differs from that of typical fibroblasts [22, 23] and although cell contacts resembling intermediate and tight junctions have been reported between embryonic fibroblasts [24] and between adult guinea pig "fibroblasts" in culture [25] they are rarely, if ever, found between adult connective tissue cells *in vivo* [24]. The relatively frequent occurrence of these contacts and of basal lamina support the suggestion that the tissue culture cells are not fibroblasts. The greater part of the extracellular material is not collagen and has some of the morphological appearances of elastic tissue. The extracellular fibrils are similar to the 100 Å microfibrillar component of elastic fibrils [26–28] which have been reported to be present during the early development of elastic tissue.

The ultrastructural characters of the cells we have described correspond well with two cells derived from the blood vascular system, the endothelium and the endothelial pericytes, both of which have specialized cell contacts and produce basal lamina. The normal appearances of these cells are described in detail by e.g. Rhodin [29] and Majno [30] and Weiner *et al.* [31] have described the ultrastructure of proliferating endothelial cells *in vivo*. Ashton and his group [32–35] have studied the embryological development, structure and function of these cells in some detail. They conclude that both cell types are derived from a common primitive vascular mesenchyme. Many other workers [36, 37] believe that the pericyte should be regarded as a multipotent primitive mesenchyme cell. Since it is located at a site in the microcirculatory bed which is capable of rapid growth during embryogenesis [38] and in wound repair [39, 40], it would not be surprising if these cells and their associated endothelial cells had a great potential for growth *in vitro*.

Although the tissue culture cells have a strong morphological resemblance to pericytes and endothelial cells, morphology alone cannot prove this derivation. We have seen no definite rod-shaped Weibel-Palade bodies [41] or cross-striated fibrils [42] similar to those found in human or rat endothelial cells.

In a preliminary study of the surface structure of one of our cell lines [2] using scanning electron microscopy, three cell types were recognized — epitheloid, spindle and giant cells — which may correspond to those which we have described. Some of the cells had surface fenestrae resembling those seen in endothelial cells. The cells examined using the scanning electron microscope are now being studied by transmission electron microscopy. Both type 1 and type 2 cells and giant cells were present in tumours which developed after reinoculation of cultured cells into mice and it seems likely that all are neoplastic, but cloning experiments to test this are in progress. The ultrastructure and histochemistry of the tumours will be described in a later paper.

**SUMMARY**

*The ultrastructure of* 11 *tumour-producing and* 13 *non-tumour producing cell lines derived from different organs of young and old C57BL and C3H mice has been examined.*

*In cultures from all organs examined (kidney, lung, bladder, tongue, heart, spleen, prostate, peritoneum and spinal cord) two predominant cell types were found. These cells had morphological characters suggesting that they may have been derived from endothelial cells and vascular pericytes. Cells in 7/11 tumour lines had cytoplasmic glycogen deposits but there were no other morphological differences between tumour and non-tumour lines from young and old mice, or between different transfer generations, or between lines derived from different mouse strains.*

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# The Lymphocytic Chalone and its Antimitotic Action on a Mouse Lymphoma *in vitro*

W. S. BULLOUGH and E. B. LAURENCE
*Mitosis Research Laboratory, Birkbeck College, University of London, Great Britain*

## INTRODUCTION

RECENT studies have revealed a remarkable uniformity in the mitotic control mechanisms of a variety of tissues. Although there are no doubt many complexities yet to be appreciated, in all the tissues so far studied mitotic control is evidently exercised basically through a negative feedback mechanism [1, 2]. In this mechanism a central role is played by a tissue specific antimitotic chemical messenger called a chalone. The tissues now known to produce and respond to such chalones are the epidermis [3, 4], sebaceous glands [5], and melanocytes [6] of the skin, the granulocytes [7] and erythrocytes [7] of the blood, and certain tissues of the liver [8], kidney [9] and lung [10]. In the blood, the mature granulocytes and erythrocytes, synthesize and release the granulocytic and erythrocytic chalones respectively. These chalones then pass to the bone marrow, where the granulocytic chalone inhibits the mitotic activity of the progranulocytes and the erythrocytic chalone inhibits the mitotic activity of the proerythrocytes.

For a long time there have been indications that, in a similar way, the circulating lymphocytes may produce their own tissue specific chalone, which passing in the blood and lymph acts to suppress the rate of lymphocyte production [11]. The questions considered here are whether such a chalone does exist, and if so whether it has the power to suppress mitotic activity in lymphocytic leukaemia. It is now known that four diverse tumours, one epidermal [12], two melanocytic [6], and one granulocytic [13] can respond by mitotic inhibition to injections of extra amounts of the appropriate chalone. Such inhibition can even lead to the total destruction of the tumour, the most dramatic results having been obtained by Rytömaa and Kiviniemi with a granuloma [14] and with a dispersed granulocytic leukaemia [15].

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## MATERIAL AND METHODS

From preliminary experiments it was found that the most convenient test material for *in vitro* studies of mitotic reactions was the L5178Y-mouse lymphoma which has a particularly high mitotic rate. Details of the growth of this tumour in adult male DBA mice have been given by Bullough and Deol [16].

About 2 weeks after subcutaneous inoculation, when the tumours had spread widely beneath the skin, the mice were killed. Small pieces, about 3 $mm^3$, were cut from the tumour edges and were maintained at 37°C in a phosphate-buffered saline medium with 0·02 M glucose and an oxygen gas phase (for details, see Bullough and Laurence [17] ). One hour was allowed to pass so that all the mitoses originally present would have passed the metaphase. Colcemid (4 μg/ml) was then added and allowed to act for 4 hr to arrest all newly-forming mitoses in the metaphase.

The various hormones and tissue extracts were added to the saline medium before the experiments began. The adrenalin was in the form of adrenalin bitartrate and in calculating the concentration (2·5 μg/ml) a correction was made for the weight of the bitartrate. The

*Table* 1. *Effects of adrenalin and hydrocortisone on the mitotic activity of mouse lymphomata* in vitro

| 4 ml culture medium with: | 1st experiment $N$ | 1st experiment M±s.e. | 1st experiment Depression | 2nd experiment $N$ | 2nd experiment M±s.e. | 2nd experiment Depression |
|---|---|---|---|---|---|---|
| Control | 23 | 46·1±1·08 | — | 16 | 30·6±2·24 | — |
| 10 μg adrenalin | 23 | 44·7±1·56 | 0 | | | |
| 10 μg hydrocortisone | 21 | 43·8±1·27 | 0 | | | |
| 10 μg adrenalin+10 μg hydrocortisone | 24 | 33·7±1·20 | 27% | 16 | 21·2±1·08 | 31% |

Average numbers of mitoses arrested by colcemid in 4 hr in unit areas of 1·5 mm² of lymphomata sectioned 7 μ thick.
$N$, number of lymphomata.

thoracic duct lymphocytes were collected over a period of several weeks from a single sheep and until required they were stored in liquid nitrogen. They were then thawed, the cells were macerated and extracted in distilled water, and the solid material was spun down at 3000 rev/min for 30 min. The resulting supernatant was then used in the experiments without further purification. The extracts of lymphomata were made from fresh material but otherwise the technique was the same. However, in this case the final supernatant also contained contaminants from the blood and the necrotic tumour tissue.

The extracts of the lymphoctyes and lymphomata were used shortly after preparation, and the amounts recorded in the tables as being added to the culture medium refer not to the actual weight but to the original wet weight of tissue or tumour from which the extracts were made. In contrast, the other extracts of tissues and tumours recorded in Table 5 were lyophilized for storage before use, and the amounts added to the culture medium refer to the dry weight of the water soluble material.

The pieces of tumour were fixed in Bouin's aqueous fluid, and were sectioned 7μ thick. The arrested mitoses were counted in section areas of 1·5 mm². In all cases $N$ is the number of separate tumours from which the pieces came.

## RESULTS

*Effects of stress hormones on lymphoma* in vitro

In previous *in vitro* studies of an epidermal carcinoma [12] and of two melanomata [6] it was found that the epidermal and melanocytic chalones respectively did not inhibit mitotic activity except in the presence of adrenalin

*Table* 2. *Effects of extracts of sheep lymphocytes, with and without adrenalin and hydrocortisone, on the mitotic activity of mouse lymphomata* in vitro

| 4 ml culture medium with: | 1st experiment $N$ | 1st experiment M±s.e. | 1st experiment Depression | 2nd experiment $N$ | 2nd experiment M±s.e. | 2nd experiment Depression |
|---|---|---|---|---|---|---|
| | without adrenalin and hydrocortisone | | | | | |
| Control | 16 | 30·6±2·24 | — | 17 | 48·0±1·07 | — |
| +100 mg extract* | 15 | 31·4±2·08 | 0 | 16 | 45·5±1·75 | 0 |
| +1000 mg extract | 15 | 27·2±1·56 | 11%† | 17 | 44·5±1·40 | 0 |
| | with 10 μg adrenalin and 10 μg hydrocortisone | | | | | |
| Control | 16 | 21·2±1·08 | — | 16 | 33·9±1·74 | — |
| +100 mg extract | 15 | 15·7±1·54 | 26% | 15 | 18·1±0·88 | 47% |
| +1000 mg extract | 17 | 16·8±0·64 | 21% | 18 | 23·6±1·54 | 30% |

Average number of mitoses arrested by colcemid in 4 hr in unit areas of 1·5 mm² of lymphomata sectioned 7μ thick. $N$, number of lymphomata.
*The weights of the extract refer not to the amount added but to the wet weight of tissue from which the extract was made.
†Not significant.

*Table 3. Effects of sheep lymphocyte extracts after heating to 100°C, with and without adrenalin and hydrocortisone, on the mitotic activity of mouse lymphomata* in vitro

| 4 ml culture medium with: | *N* | M±s.e. | Depression |
|---|---|---|---|
| | without adrenalin and hydrocortisone | | |
| Control | 8 | 45·4±1·07 | — |
| +100 mg normal extract* | 9 | 40·9±1·52 | 0 |
| +100 mg heated extract | 9 | 40·8±1·63 | 0 |
| +1000 mg normal extract | 9 | 41·4±2·10 | 0 |
| +1000 mg heated extract | 10 | 43·1±1·71 | 0 |
| | with 10 μg adrenalin and 10 μg hydrocortisone | | |
| Control | 9 | 30·4±2·29 | — |
| +100 mg normal extract* | 9 | 19·2±0·99 | 43% |
| +100 mg heated extract | 8 | 32·4±1·55 | 0 |
| +1000 mg normal extract | 8 | 16·1±1·08 | 47% |
| +1000 mg heated extract | 9 | 31·7±1·76 | 0 |

Average numbers of mitoses arrested by colcemid in 4 hr in unit areas of 1·5 mm² of lymphomata sectioned 7 μ thick.
*N*, number of lymphomata.
*The weights of the extract refer not to the amount added but to the wet weight of tissue from which the extract was made.

and a glucocorticoid hormone. Therefore the reactions of the lymphoma to these two hormones, separately and together, were investigated. The concentrations used (2·5 μg/ml) were those previously found to be effective with the other tumours. The results of two separate experiments are in Table 1.

The conclusions are that, in the concentrations used, neither adrenalin nor hydrocortisone by themselves had any antimitotic action on the lymphoma cells, but that when these hormones were present together they induced a significant mitotic depression of about 30%. This result is closely similar to those previously obtained with the epidermal carcinoma [12] and the melanomata [6], when it was concluded that the depression was caused by the co-operative action of the two hormones with the relatively small amount of chalone remaining in the tumour cells.

*Effects of lymphocyte extracts on lymphoma* in vitro

The search for a lymphocytic chalone began with the aqueous extract of sheep lymphocytes which was tested for its action alone and in combination with the two stress hormones. The results of two separate experiments are in Table 2.

The conclusions are that the extract alone had no significant antimitotic action; that the two stress hormones induced the usual mitotic depression; and that the extract, together with the stress hormones, induced a dose-dependent mitotic depression which exceeded that caused by the stress hormones by from 20% to 50%. On the assumption that this effect was due to the presence of a lymphocytic chalone, these results are again closely similar to those obtained with the epidermal chalone on an epidermal carcinoma and with the melanocytic chalone on two melanomata. In all these cases the chalone was dependent for its antimitotic action on the co-operation of the two stress hormones. In all cases, too, the chalones, although tissue and tumour specific, were not species specific.

On present information it appears that the epidermal chalone is a glycoprotein and it is known that it [18] and other chalones are destroyed by heating to 100°C. The experiments were therefore repeated using a sheep lymphocyte extract that had been kept for 10 min at 100°C. The results are in Table 3.

The conclusion is that antimitotic activity of the lymphocyte extract is destroyed by heating.

*Effects of lymphoma extracts on lymphoma* in vitro

In the previous tumour studies it was found that epidermal chalone could be extracted from the epidermal carcinoma [12] and that the melanocytic chalone could be extracted from the two melanomata [6], although in all cases the cellular chalone concentration was evidently far below normal. Experiments were therefore carried out in which extracts of the lymphoma

*Table* 4. *Effects of extracts of mouse lymphoma, with and without adrenalin and hydrocortisone, on the mitotic activity of the same lymphoma* in vitro

| 4 ml culture medium with: | *N* | M±s.e. | Depression |
|---|---|---|---|
| | without adrenalin and hydrocortisone | | |
| Control | 9 | 50·3±1·37 | — |
| with 10 mg extract* | 8 | 53·8±1·32 | 0 |
| with 100 mg extract | 9 | 50·8±0·61 | 0 |
| with 1000 mg extract | 7 | 42·3±1·09 | 16% |
| | with 10 μg adrenalin and 10 μg hydrocortisone | | |
| Control | 8 | 38·1±0·97 | — |
| with 10 mg extract* | 9 | 23·1±1·26 | 39% |
| with 100 mg extract | 9 | 19·0±0·57 | 50% |
| with 1000 mg extract | 9 | 10·8±1·24 | 72% |

Average numbers of mitoses arrested by colcemid in 4 hr in unit areas of 1·5 mm² of lymphomata sectioned 7 μ thick.
*N*, number of lymphomata.
*The weights of the extract refer not to the actual amount added but to the wet weight of tumour from which the extract was made.

were tested for their activity against the mitotic activity of the same lymphoma. The results are in Table 4.

The conclusions are that the lymphoma extract used alone had no specific antimitotic activity and that the slight depression induced by the highest concentration of this extract was probably due to the non-specific action of some contaminant, perhaps from the blood or necrotic tumour tissue; that the adrenalin and hydrocortisone caused the usual mitotic depression; and that in the presence of the two stress hormones the lymphoma extract induced a deep mitotic depression which was much greater than could be attributed to the non-specific action already mentioned. Clearly these results are consistent with the presence of a lymphocytic chalone in the lymphoma extracts.

*Tissue and tumour specificity*

One characteristic of a chalone is the strict tissue specificity of its antimitotic action. Thus extracts of other tissues and tumours should have no antimitotic action on the lymphoma

*Table* 5. *Effects of extracts of various tissues and tumours, with and without adrenalin and hydrocortisone, on the mitotic activity of mouse lymphoma* in vitro

| 4 ml culture medium with | No adrenalin or hydrocortisone | | | 10 μg adrenalin+ 10 μg hydrocortisone | | |
|---|---|---|---|---|---|---|
| | *N* | M±s.e. | Depression | *N* | M±s.e. | Depression |
| Control | 6 | 40·8±1·28 | — | 7 | 33·0±1·36 | — |
| 10 mg pig lung extract* | 4 | 42·8±0·56 | 0 | 6 | 34·0±1·44 | 0 |
| 10 mg pig skin extract | 5 | 40·1±2·50 | 0 | 6 | 31·8±2·46 | 0 |
| 10 mg mouse-lung tumour extract | 4 | 42·6±0·71 | 0 | 6 | 30·8±0·56 | 0 |
| 10 mg human-lung tumour extract | 5 | 39·8±1·17 | 0 | 4 | 36·0±0·36 | 0 |
| 10 mg Harding-Passey melanoma extract | 6 | 44·5±2·02 | 0 | 5 | 35·6±1·60 | 0 |

Average numbers of mitoses arrested by colcemid in 4 hr in unit areas of 1·5 mm² of lymphomata section 7 μ thick.
*N*, number of lymphomata.
*In the case of each extract the weight added was that of water soluble material which had been lyophilised for storage.

*Table 6. Effects of extracts of sheep lymphocytes on the mitotic activity of mouse epidermis* in vitro

| Time after start of experiment *in vitro* | | | |
|---|---|---|---|
| 1–5 hr | | 5–9 hr | |
| Control M±s.e. | 100 mg extract*/4 ml M±s.e. | Control M±s.e. | 100 mg extract*/4 ml M±s.e. |
| 10·9±0·34 | 10·8±0·20 | 10·4±0·34 | 9·4±0·51 |

Average numbers of mitoses arrested by colcemid in 4 hr in unit lengths of 1 cm of ear epidermis section 7 μ thick.
*N*, number of mice=5.
*The weight of the extract refers not to the amount added but to the wet weight of tumour from which the extract was made.

and extracts of the lymphoma should have no antimitotic action on other tissues. A number of tissue and tumour extracts were therefore tested against the lymphoma, and of these it was known that the skin extracts contained both the epidermal and melanocytic chalones, that the melanoma extract contained the melanocyte chalone, and that in all probability [10] the lung extract contained at least one lung tissue chalone. The results are in Table 5.

From these it is clear that, in the doses used, none of the extracts exerted any antimitotic action on the lymphoma whether or not the stress hormones were also present.

In the reverse situation the lymphoma extract was tested against the mitotic activity of mouse epidermis *in vitro*, but the design of this experiment had to be different from that of the experiments described above. Normal epidermal cells contain so much epidermal chalone that if the two stress hormones are added at the concentration of 2·5 μg/ml the antimitotic action of the three substances together becomes so powerful that mitotic activity almost ceases. Furthermore it has been established [3, 4] that when epidermis is newly removed from a mouse it contains such adequate amounts of endogenous adrenalin and glucocorticoid hormone that no more need be added to the culture medium to enable the cells to respond by mitotic inhibition to extra epidermal chalone. However, after about 5 hr *in vitro* when the endogenous adrenalin has been lost, the epidermal cells cease to respond to the extra chalone.

For these reasons the lymphocyte extract was added alone to the culture medium and the epidermal mitotic rate was measured during 1–5 hr when the endogenous adrenalin was present and during 5–9 hr when the adrenalin had been lost. The results are in Table 6.

The conclusion is that the lymphocyte extract was powerless to suppress epidermal mitosis either in the presence of adrenalin and a glucocorticoid hormone or later when little or no adrenalin remained.

## DISCUSSION

While the present work was in progress Moorhead *et al.* published closely parallel results to show that crude water extracts of pig lymph nodes contain some substance which inhibits the entry into DNA synthesis and into mitosis of phytohaemagglutinin-stimulated human lymphoctyes *in vitro* [19] and of human lymphocytic leukaemic cells *in vitro* [20]. They suggest that the active substance may be a protein of MW 50,000–75,000. Taken together with the present evidence, this shows that a mitotic inhibitor is present in pig lymph nodes, sheep lymphoctyes, and mouse lymphoma, and that this inhibitor acts in both the pre-S phase and the pre-M phase in both normal and leukaemic lymphocytes.

It is obviously important to obtain details of the physiological and chemical characteristics of this inhibitor, and the first question is whether it is a chalone. The characteristics of a typical chalone are: it is water soluble and heat labile; it is synthesized by the same tissue on which it acts to inhibit cell division; it is not species specific; and its action is commonly strengthened in the presence of adrenalin and a glucocorticoid hormone. The present evidence is that the lymphocytic mitotic inhibitor conforms to these rules and therefore that it is probably the lymphocytic chalone.

The lymphocyte population of the body is maintained from day to day at a level at which cell gain by mitosis is exactly balanced by cell loss through cell death and through cell differentiation into one of the antibody-producing tissues. The presumption now is that this is also the point at which the amount of chalone released from the post-mitotic lympho-

cytes is adequate to limit to the correct degree the mitotic activity of the lymphoid centres. If an animal is subjected to chronic stress the antimitotic power of the chalone will be strengthened by the raised stress hormone concentration, the rate of cell gain will fall below the rate of cell loss, and the lymphocyte population will be reduced until it reaches a new point of balance at a lower level. This is evidently the explanation of the well-known antilymphocyte action of the glucocorticoid hormones.

The high mitotic activity shown by human lymphocytic leukaemic cells [20] and by mouse lymphoma cells, combined with their mitotic inhibition when treated with extra amounts of chalone, suggests that one important characteristic of these cells is a subnormal intracellular chalone concentration. In this they would then resemble the granulocytic leukaemic cells of both rats [13, 15] and men [15] described by Rytömaa and Kiviniemi. In the rat, if a sufficiently high concentration of the granulocytic chalone can be artificially maintained for a sufficient length of time, a granuloma [14] or a dispersed granulocytic leukaemia [15] can be suppressed and even destroyed. The opportunity now exists for a similar attack on a lymphoma or a dispersed lymphocytic leukaemia using the lymphocytic chalone. However, at the moment such *in vivo* experiments present serious practical difficulties. To induce the regression of a tumour large amounts of chalone must be repeatedly injected, and, if the chalone extracts are only crude, the even larger amounts of foreign proteins that are also present can be lethal. Techniques for the production of sufficiently large quantities of sufficiently pure chalones have not yet been devised although some partial success has been achieved with the granulocytic [14, 15] and epidermal [21] chalones.

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## SUMMARY

*The mitotic activity of L5173Y-mouse lymphoma* in vitro *is inhibited by extracts of normal sheep lymphocytes and of the lymphoma cells themselves. The inhibitory substance is water soluble and heat labile, is evidently tissue-specific, is not species-specific, and its action is only apparent when adrenalin and hydrocortisone are present. These are the common characteristics of chalones and it appears that this inhibitor is the lymphocytic chalone, which normally acts to control the rates of lymphocyte production.*

*The evidence suggests that the lymphoma cells contain an inadequate concentration of this chalone, and when the chalone concentration is artificially raised their mitotic activity is inhibited. It has similarly been shown that the division of granulocytic leukaemic cells from rat and from man can be inhibited by a raised concentration of the granulocytic chalone.*

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# Heterotransplantation of Human Cancers to Animals by Means of Diffusion Chambers

T. P. EVGENJEVA

*The Severtzov Institute of Evolution Morphology and Ecology of Animals, Academy of Sciences, Moscow, U.S.S.R.*

## INTRODUCTION

EXCEPT for the work of Toolan in the subcutaneous space of conditioned animals [1, 2] and of Patterson with the hamster cheek pouch [3], transplantation studies with human tumors are few in number. Recently it was found that Sander's observation chambers could be conveniently used to measure the growth and response to cytotoxic drugs of human tumours *in vivo* [4].

The present paper describes an attempt to maintain human tumours by means of diffusion chambers. This technique was first described by Algire *et al.* in 1954 [5].

## MATERIAL AND METHODS

We studied the growth of human cancers in diffusion Millipore chambers, which were transplanted into laboratory animals. Carcinomas of the stomach (primary with metastases to regional lymph nodes), adenocarcinomas of the breast, carcinoma of the lung and carcinoma of the ovary were used. Fresh tissue was obtained from the operating room. Most of our experiments have been done with a transit time from patient to animal of under 5 hr. Small pieces (not exceeding 0·5 $mm^3$) of tumour tissue were placed in Millipore diffusion chambers. The chambers were 10 mm o.d. and 6 mm i.d. The membranes had an average pore diameter of 0·1–0·3 μ. The chambers were fabricated as reported earlier [6]. The diffusion chambers were then implanted intraperitoneally or subcutaneously in the designated rats and Syrian hamsters. Tissue from each tumour was transplanted to 15–20 diffusion chambers at a time. The data of these experiments are summarized in Table 1. At specific times (3, 7, 10, 14 days) the chambers were withdrawn, the filters were fixed and total preparations were stained with Carrachi hematoxylin. The cytologic characteristics, particularly those used for diagnosis, were described and documented for each tumour tissue in Figs. 1–12.

Uptake of $^3$H-thymidine was demonstrated in the filters by means of an autoradiographic technique. $^3$H-thymidine was introduced into animals 2 hr before fixing the chambers. Autoradiographs of filters were obtained using the dipping-film technique.

## RESULTS

Carcinomas of stomach were transplanted successfully in diffusion chambers. The tendency of epithelial cells to adhere to each other resulted in the formation of cell aggregates containing a recognizable histological pattern. Cytoplasm was not well spread on the filter and cells appeared more cohesive, growing in clumps and clusters (Figs. 1–3). The shape of cells varied from oval to cylindrical shaped. The majority of labelled nuclei were in epithelial cells (Fig. 3.)

A metastatic lymph node was a particularly good source of tissue for transplantation, but these transplants were characterized by poorly differentiated cells (Fig. 4). Two cytologic cell

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*Table* 1. *Summary of data on transplantation of human cancers in diffusion chambers*

| Donors | | Recipients | | |
|---|---|---|---|---|
| Site | Tumour | No. of tumour | Species | No. of diffusion chambers with tumour / No. of animals with diffusion chambers |
| Stomach | Carcinoma | 2 | rat | 14/14 |
| | | | hamster | 12/15 |
| | Metastasis in the lymph node | 2 | rat | 5/5 |
| | | | hamster | 4/5 |
| Breast | Adenocarcinoma | 4 | rat | 28/30 |
| | | | hamster | 20/22 |
| Lung | Bronchopulmonary carcinoma | 1 | rat | 9/10 |
| | | | hamster | 10/10 |
| Ovary | Serous cystadenocarcinoma | 1 | rat | 10/10 |
| | | | hamster | 5/5 |

types were present. The major population consisted of large epithelium-like cells with well-spread cytoplasm (arrows *A*). The nuclei showed variation in size and shape as well as in basal orientation. Second type was a small macrophage-like cells with densely staining nuclei and scant cytoplasm (arrows *B*).

Transplants of breast cancers (adenocarcinoma, Fig. 5) grew well in diffusion chambers. The general morphology was similar to that of the original. The cells were quite uniform in size and shape with well-spread cytoplasm and prominent nucleoli. At the 7th day the cells had an original arrangement with obvious gland formation (Figs. 6, 7). Mitotic index was low, it is clear therefore that an upward movement may have played a part in the rapid increase in epithelial thickness during the first 7 days. Relatively little stroma was present.

Carcinoma of the lung grew extensively in the diffusion chambers. The histological pattern was that of a bronchopulmonary carcinoma with lymph nodes involved by tumour (Fig. 8). When tumour cells were placed in a diffusion chamber they assumed morphologic patterns similar to those of original tumour. Microscopic examination of transplants revealed masses of large cells with finely granular cytoplasm (Fig. 9). The cells were growing in monolayer sheets, but intercellular spaces were distinct. The nucleus generally had one or two nucleoli.

The ovarian tumour implants were characterized by the formation of pseudofollicular structures or cystic areas (Figs. 10–12). The cells were either round or oval shaped, cytoplasm of these cells was vacuolated. Nuclei were large and pale and nucleoli became more prominent. The distribution of the $^{3}$H-labelled cells on the filter was erratic (Fig. 12, arrows).

## DISCUSSION

We are reporting 8 human cancers successfully transplanted to hamsters and to rats by means of diffusion chambers. The histological examination after the observation period showed that human cancer implants contained viable tumour cells. Absence of necrosis was characteristic. Radioautographic data demonstrated that $^{3}$H-thymidine was incorporated in the epithelial cells.

The techniques available for the maintenance of human tumours in a foreign host are relatively few (1–4·7). X-radiation and cortisone may be an influence in the property of heterotransplanted cancer cells.

From consideration of the results obtained during the present study, it is evident that human tumours maintained their histological identity after transplantation to the animals by means of diffusion chambers. The used technique also has the great advantage of requiring very simple conditions.

The fact that human cancers grew progressively when transplanted to the diffusion chambers implanted into laboratory animals makes it a potential model for evaluating new drugs and other forms of studies with human tumours.

**Acknowledgments**—I am grateful to Prof. V. N. Geracimenko (Institute of Experimental and Clinical Oncology of the U.S.S.R. Academy of Medical Sciences) for giving all human material.

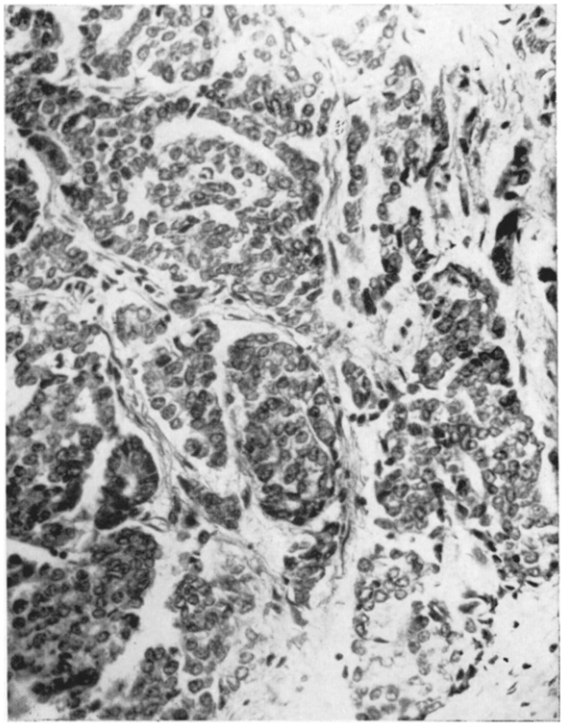

*Fig.* 1. *Original carcinoma of the stomach, biopsy, section.*

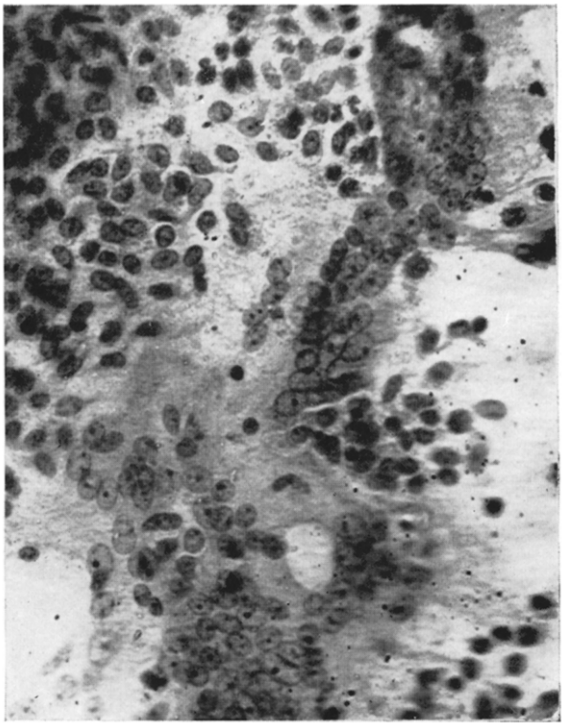

*Fig.* 2. *Tissue of the stomach carcinoma after* 14 *days of cultivation in a diffusion chamber, total preparation.*

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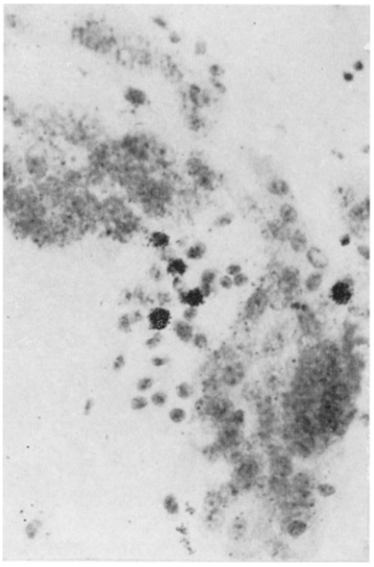

*Fig. 3. Autoradiograph of a typical aggregate obtained after 10 days of cultivation in a diffusion chamber. total preparation.*

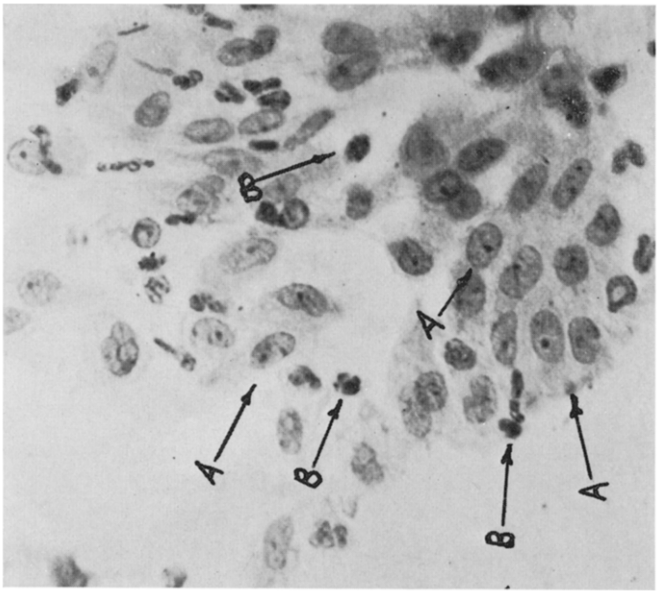


*Fig. 4. Metastasis of stomach carcinoma to regional lymph node 7th day cultivation in a diffusion chamber total preparation.*

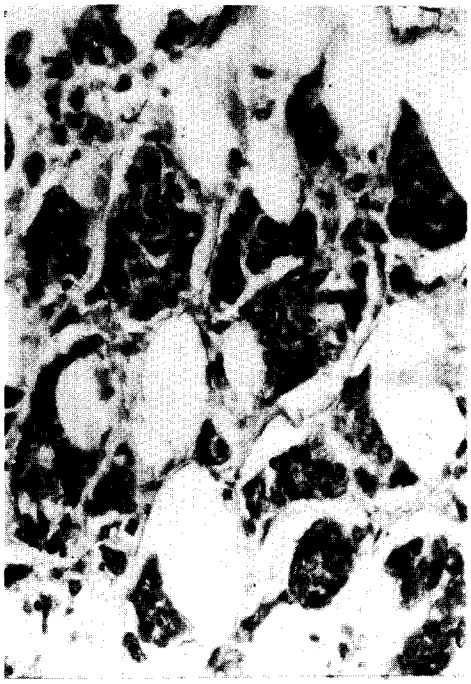

*Fig. 5. Adenocarcinoma of breast. Biopsy from primary tumour, section.*

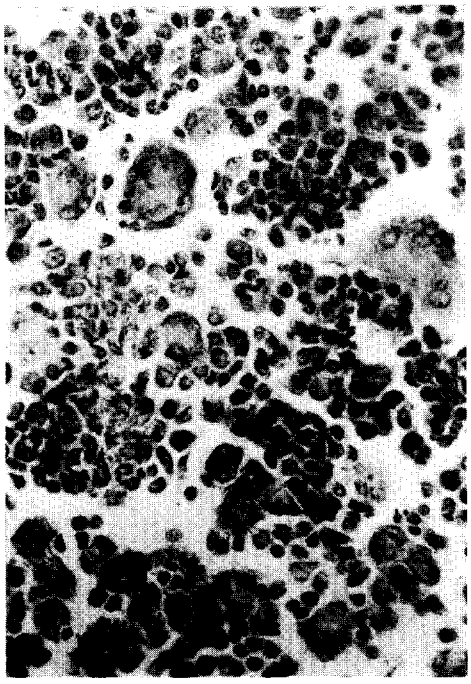

*Fig. 6. Tissue of this tumour after 7 days of cultivation in a diffusion chamber, total preparation.*

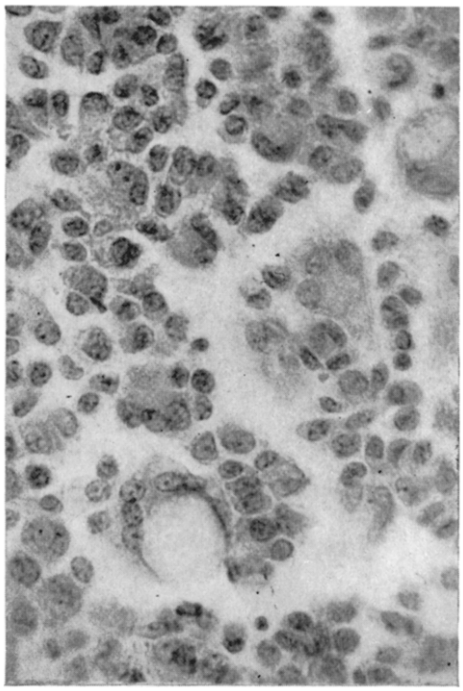

*Fig.* 7. *Epithelial cell layer.* 14*th day cultivation adenocarcinoma of breast in diffusion chamber, total preparation.*

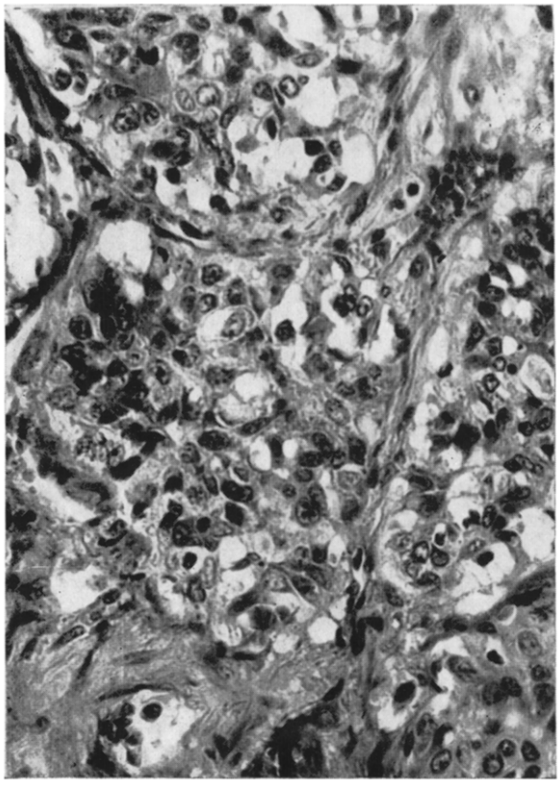

*Fig.* 8. *Bronchogenic carcinoma, biopsy, section.*

*Fig.* 9. *Tissue of this tumour after* 7 *days of cultivation in a diffusion chamber, total preparation.*

*Fig.* 10. *Original carcinoma of the ovary, biopsy, section.*

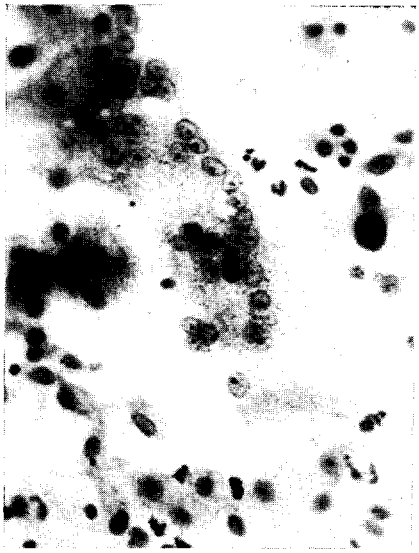

*Fig.* 11. *Tissue of this tumour after* 7 *days of cultivation in a diffusion chamber, total preparation.*

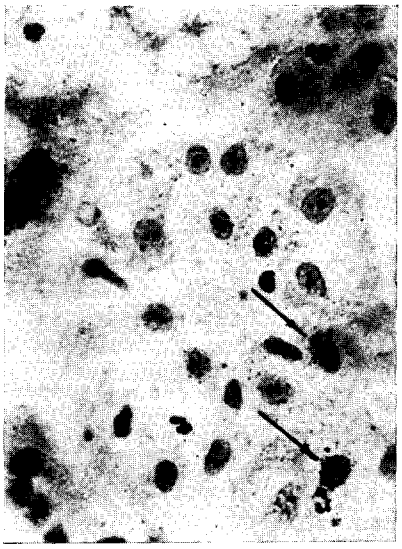

*Fig.* 12. *Autoradiograph of ovarian carcinoma, obtained after* 10 *days of cultivation in a diffusion chamber, total preparation.*

## SUMMARY

1. *The tumours grew successfully in diffusion chambers which were implanted into animals (rats and Syrian hamsters).*
2. *In this condition the tumour tissue retained their histological specificity.*


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# The Histopathology of Tumors Induced by PARA-adenovirus 7 Transformed Cell Clones

S. PAULUZZI,[1] R. RIBACCHI,[2] R. FRONGILLO[1] and M. P. ZEPPA[1]

[1]*Institute of Infectious Diseases and* [2]*Institute of Morbid Anatomy and Histology, Division of Cancer Research, Perugia University, Italy*

## INTRODUCTION

HUMAN and simian oncogenic adenoviruses induce undifferentiated tumors, with small, densely packed, embryonal-type cells and a uniform pattern in hamsters [1]. It is difficult to trace their normal precursor cells. These neoplasms are labelled according to different authors as "undifferentiated carcinomas" [2], or "small-cell sarcomas" [1].

Only Neiders *et al.* [3] obtained typical liver adenocarcinomas after intravenous injection of adenovirus 12 into newborn hamsters.

SV40 tumors on the other hand are spindle cell sarcomas with some pleomorphic areas [1]. SV40 also induces ependymomas and tumors of choroid plexus in hamsters [4–6] and in Mastomys [7]. Hamster cells transformed *in vitro* by SV40 produced transplantation tumors recognized as carcinosarcomas, adenocarcinomas, epidermoid carcinomas [8–11] and astrocytomas [12].

PARA-adenovirus hybrids are formed by a mixed population: (a) a *human adenovirus* which is defective in monkey cells and replicates only in the presence of SV40 or PARA-particles; (b) *PARA-particles* (particles aiding replication of adenovirus), which have a defective SV40 genome encased in an adenovirus capside and are able to synthesize SV40 T antigen but not SV40 virions nor its structural subunits. PARA-particles can multiply in monkey kidney cells only in the presence of an adenovirus which furnishes the coat protein [13].

The PARA-adenovirus hybrids are oncogenic for the newborn hamsters, even when the adenovirus component is not by itself oncogenic [14]. PARA-induced tumors have the microscopic features either of adenovirus tumors or of SV40 tumors; moreover the same tumor may show both aspects ("mixed tumors") [15, 16].

SV40 T antigen is constantly present in all these tumors: undifferentiated, adenovirus-type, SV40 spindle cell sarcomas and mixed tumors. On the contrary, the adenovirus T antigen (s) is present only in the undifferentiated and mixed tumors [15, 17]. The neoplasms containing both adenovirus and SV40 tumor antigens appear earlier than those carrying only SV40 T antigen [17]. When inoculated intracerebrally into newborn hamsters PARA-adenovirus 7 induces ependymomas [18]. Hamster cells transformed *in vitro* by PARA-adenovirus hybrids produce transplantation tumors, recognized as carcinosarcomas, with areas of adenocarcinoma [19].

The hypothesis may be forwarded that PARA-adenovirus hybrids induce mixed tumors in the hamster in two ways: either they cause different cell types in the same neoplasm, or a single tumor cell, which differentiates into several histologic–antigenic patterns, is formed. To investigate this problem, clonal cell lines were obtained from tumors induced by PARA-adenovirus 7 and their antigenic features were

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verified. The histopathologic pattern of the tumors induced by these cell clones was compared with that of adenovirus or SV40 tumors.

## MATERIAL AND METHODS

*Viruses*

Two PARA-adenovirus 7 clonal lines were used: P10 (60) and P10 (189), both were obtained from the LL strain of adenovirus 7 (20) (PARA-adenovirus 7 hybrid).

The LL strain was plaque-purified twice onto GMK cells in the presence of helper adenovirus type 7. P10 (60) is the progeny of a terminal PARA-plaque.

The second PARA-adenovirus 7 clonal line was obtained from the LL strain by transcapsidatin 8 [13] it to adenovirus 21, adenovirus 6 and again to adenovirus 7. The PARA-adenovirus 7 transcapsidant was plaque-purified twice onto GMK cells in the presence of helper adenovirus type 7. P10 (189) is the progeny of a terminal PARA-plaque.

The adenovirus type 12, strain 6830, was obtained through the courtesy of Dr. Fred Rapp. It was propagated in HeLa and HEK cells and titrated in plaque-forming units onto HEK monolayers.

*Adenovirus and SV40 tumor cell lines*

The H-50 cells were derived from a hamster tumor induced by SV40 [21] and obtained through the courtesy of Dr. Satvir S. Tevethia (at more than 100 passages *in vitro*) and were further propagated in our laboratory for 39 passages. These carry nuclear SV40 T antigen.

The HBT cells were derived from a hamster brain tumor induced by intracerebral inoculation of simian adenovirus 7 (SA7). Their characteristics were described in a previous paper [22]. HBT carry adenovirus SA7 T antigen in a cytoplasmic location and are devoid of SV40 T antigen.

The adenovirus 7 cells (henceforth called strain: "Ad 7") were derived from a hamster subcutaneous tumor induced by adenovirus type 7 and were obtained from the Flow Laboratories Inc., Irvine, Scotland. They were further propagated in our laboratory for 18 passages.

H-50, HBT, Ad 7 cells were propagated in Eagle MEM double strength, 1·8 mM $CaCl_2$, 10% fetal calf serum.

*Immunofluorescence techniques*

Cells were grown on 16 mm cover glasses in petri dishes and held in an atmosphere containing 5% $CO_2$. When harvested, cells were washed three times with tris-(hydroxymethyl) aminomethane (tris) buffer (pH 7·4), air dried, and fixed with acetone for 3 min at room temperature. They were stained at room temperature by reacting them for 30 min with sera from hamster bearing tumors induced by SV40, adenovirus 7, adenovirus 12, simian adenovirus 7; thereafter they were washed three times with tris buffer. The cells were then reacted with fluorescein-labelled rabbit globulin anti-hamster globulin for 30 min, washed three times again with tris buffer, air dried and mounted in Elvanol. The slides were viewed with a Zeiss fluorescence microscope.

The tumors induced by adenovirus 12 in newborn hamsters and newborn C3H/He mice were sectioned at 6μ in the Tissue-Tek microtome-cryostat and the sections were thereafter treated following the same immunofluorescent procedure.

*Animal experiment*

Random-bred Syrian hamsters* and inbred C3H/He mice† were inoculated subcutaneously with $10^5$–$10^6$ plaque-forming units of adenovirus 12 within 24 hr after birth. The animals were weaned 3 weeks later and checked weekly for tumor development. When tumors measured greater than 20 mm dia., the animals were bled out and the tumors excised.

Weanling Syrian hamsters* were inoculated subcutaneously with $0·4$–$1·0 \times 10^7$ tumor cells in 0·2 ml volumes. Thereafter they were observed at weekly intervals and the tumor diameters were measured with calipers.

One piece of each tumor was fixed in neutral 10% formaline, dehydrated in alcohol, embedded in paraffin and sectioned at 5μ. The sections were stained with hematoxylin and eosin, Van Gieson, Mallory and Gomori stainings.

## RESULTS

*Derivation of clonal cell lines of PARA-adenovirus 7 tumors*

Two clonal cell lines were obtained from PARA-adenovirus 7 tumors, the 819/R/A2a and the 928/R/B/Str lines.

*819/R/A2a line.* $2 \times 10^5$ plaque-forming units of PARA-adenovirus 7 P10 (60), titrated in GMK cells in the presence of helper adenovirus 7 [23], were inoculated subcutaneously in 0·1 ml volume into hamsters of the Lakeview Hamster Colony (Newfield, N.J.) within 24 hr after birth. One of the early tumors, with a latent period of less than 10 weeks [17], was tryp-

*Morini Hamster Colony, Reggio Emilia, Italy.
†A.R.S.A.L., Rome, Italy.

*Table* 1. *Antigenic markers of the cell lines and tumors examined*

| Cell lines | Transforming virus | SV40 T antigen | Adenovirus T antigen |
|---|---|---|---|
| 819/R/A2a | PARA-adenovirus 7 | + | ● |
| 928/R/B/Str | PARA-adenovirus 7 | + | + |
| HBT | SA 7 | ● | + |
| Ad 7 | Adenovirus 7 | ● | ● |
| H-50 | SV40 | + | ● |
| Tumors | | | |
| C3H/Ad 12 | Adenovirus 12 | ● | + |
| Hamster/Ad 12 | Adenovirus 12 | ● | + |

sinized 94 days after the virus inoculation and the cells were grown in Eagle MEM double-strength with 0·1 mM $CaCl_2$, 10% fetal calf serum. At the fourth passage the cells were cloned twice, the first time by plating them onto a feeder-layer of hamster embryo cells irradiated with 2000 R. Each time a single colony was picked up with a Pasteur pipette and the cell line could be further propagated *in vitro*.

*928/R/B/Str line.* $3{\cdot}8\times10^5$ plaque-forming units of PARA-adenovirus 7 P10 (189) were inoculated subcutaneously in newborn hamsters of the Lakeview Hamster Colony. One of the early tumors, with a latent period of less than 10 weeks, was excised and trypsinized 88 days after virus inoculation. The cells were grown in the medium indicated above and after 5 passages they were cloned in a soft agar (0·35%) medium. A colony was picked up and propagated onto a feeder-layer of hamster embryo cells irradiated with 2000R. After this passage, it was easily possible to grow this clonal cell line *in vitro*.

*Antigenic analysis of the cell strains and tumors*

SV40 T and adenovirus T antigens were looked for by the immunofluorescence techniques. As far as the adenovirus T antigen(s) is concerned, the three antisera used (anti-adenovirus 7, 12, SA7 T antigen) cross-reacted to a greater extent. The results are correlated in Table 1.

Both the PARA-adenovirus 7 cell lines 819/R/A2a and 928/R/B/Str contained nuclear SV40 T antigen, as did the H-50 cells (SV40-transformed), with a frequency approaching 100%. Only the 928/R/B/Str cells showed adenovirus T antigen(s) in a cytoplasmic location; tiny filaments of antigen were observed in almost all the cells. The same antigenic pattern and location were found in the HBT cells (SA7-transformed) and in adenovirus 12 C3H mouse and hamster tumors.

The PARA-adenovirus 7 cell line 819/R/A2a did not contain adenovirus T antigen(s), as did the adenovirus 7 tumor cells. Moreover hamsters bearing tumors induced by these two cell lines did not show antibodies against adenovirus T antigen by the immunofluorescent test on cells vegetatively infected by adenovirus type 7.

The antigenic pattern of the PARA-adenovirus 7 cell lines was checked at different passages (Table 2) and was found steady.

*Production of subcutaneous tumors by PARA-adenovirus 7, HBT, Ad 7 and H-50 cells*

$0{\cdot}4$–$1{\cdot}0\times10^7$ cells were inoculated subcutaneously in weanling Syrian hamsters. As shown in Table 3, tumors developed in almost all animals after a fairly short latent period.

*Production of subcutaneous tumors by injection of adenovirus type 12*

Adenovirus type 12 was inoculated subcutaneously in newborn C3H/He mice and Syrian hamsters within 24 hr after birth. Latent periods and tumor yields are shown in Table 4.

*Table* 2. *Antigenic analysis of the PARA-adenovirus 7 cell clones at different passages* in vitro *by the immunofluorescent test*

| Cell strain | Passage No. | SV40 T antigen | Adenovirus T antigen |
|---|---|---|---|
| 819/R/A2a | 8th | + | ● |
| | 50th | + | ● |
| 928/R/B/Str | 6th | + | + |
| | 41st | + | + |

*Table 3. Development and histologic pattern of the transplanted tumors*

| Cells inoculated | *In vitro* passage No. | Cells inoculated per animal | Latent period (days) | Animals with tumors/animals inoculated | Tumor histopathologic pattern |
|---|---|---|---|---|---|
| 819/R/A2a | 38th | $10^7$ | 7–21 | 11/11 | adenovirus |
| 928/R/B/Str | 39th | $4\times10^6$ | 7–28 | 5/11 | adenovirus |
| HBT | 59th | $10^7$ | 7–21 | 11/12 | adenovirus |
| Ad 7 | >18th | $10^7$ | 7–21 | 13/16 | adenovirus |
| H-50 | >39th | $10^7$ | 7–21 | 12/12 | SV40 |

*Table 4. Development and histopathology of the primary tumors*

| Virus inoculated | PFU per animal | Animal species | Latent period (days) | Animals with tumors/ animals inoculated | Tumor histopatho-logic pattern |
|---|---|---|---|---|---|
| Adenovirus 12 | $8{\cdot}5\times10^5$ | C3H/He mice | 159–179 | 3/13 | adenovirus |
| Adenovirus 12 | $1{\cdot}3\times10^5$ | Syrian hamster | 74–144 | 5/12 | adenovirus |

The number of tumors in C3H mice was lower than in hamsters and the latent period longer. One of the adenovirus 12 tumors in a C3H mouse regressed spontaneously after several weeks.

*Histopathology of transplanted and primary tumors*

Transplanted and primary adenovirus and PARA-adenovirus 7 tumors were softer and more friable than tumors induced by H-50 cells. All tumors were poorly invasive. Lung metastases were observed in only 2 out of 13 Ad 7 tumors. In hamster tumors induced by adenoviruses and by PARA-adenovirus 7 (both primary and transplanted), areas of necrosis and hemorrhage were prominent, and sometimes involved almost the whole growth even when they were very small. Extensive necrotic areas were also shown in H-50 tumors. Necrosis was more restricted in primary tumors induced in C3H mice by adenovirus 12.

Histologically, two different patterns were observed. The adenovirus and PARA-adenovirus tumor group showed a common morphologic picture with some individual variations. They were basically formed of small fairly uniform spindle cells which contained an oval or irregular round nucleus rich in chromatin of granular type and scanty in eosinophylic cytoplasm (Figs. 1a, b, c and d). The spindle cells were arranged radially in a few (Fig. 1a and b) or several layers (Fig. 1c and d) around a tiny bundle of connective tissue containing a vascular channel and they acquired the histological picture of trabecular type with pseudorosette formation (Fig. 1a, b and c). In the central part of the tumors the neoplastic elements were more irregularly round and were located around blood capillaries which were often ectasic (Fig. 1e).

Giant cells of different sizes were present in each tumor (Fig. 1f, g and h). They were formed by two or several round or irregular nuclei, located in different layers in the central part of the cell, and by scanty eosinophylic cytoplasm. Mitosis were usually frequent. A number of foci of necrosis were always present. Mallory and Gomori stainings showed tiny and rare collagen and argirophilic fibers only in the stromal tissue (Fig. 2a). Trabecular arrangement of neoplastic cells and pseudorosette formation were more differentiated and diffuse in adenovirus 7 and 12 hamster tumors (Fig. 1a) than in PARA-adenovirus 7 tumors (Fig. 1b, c and d). Simian adenovirus 7 tumors differed from those previously described for a less differentiated growth and a smaller number of pseudorosette formation (Fig. 2b). Giant cells of adenovirus type were also present but were smaller in size.

H-50 induced tumors (SV40 transformed cells) showed a typical structure of mesenchimal spindle cell sarcomas. They were formed by spindle cells containing an oval or vesicular nucleus and by an abundant eosinophilic cytoplasm (Fig. 2c). The chromatin was clumped at nuclear membrane. Nucleoli were well developed and cellular borders slightly distinct. Neoplastic cells were arranged in bundles of different sizes variably oriented. Mitoses were numerous and sometimes atypical, and characteristic SV40 giant cells were present in some tumors. These were formed by

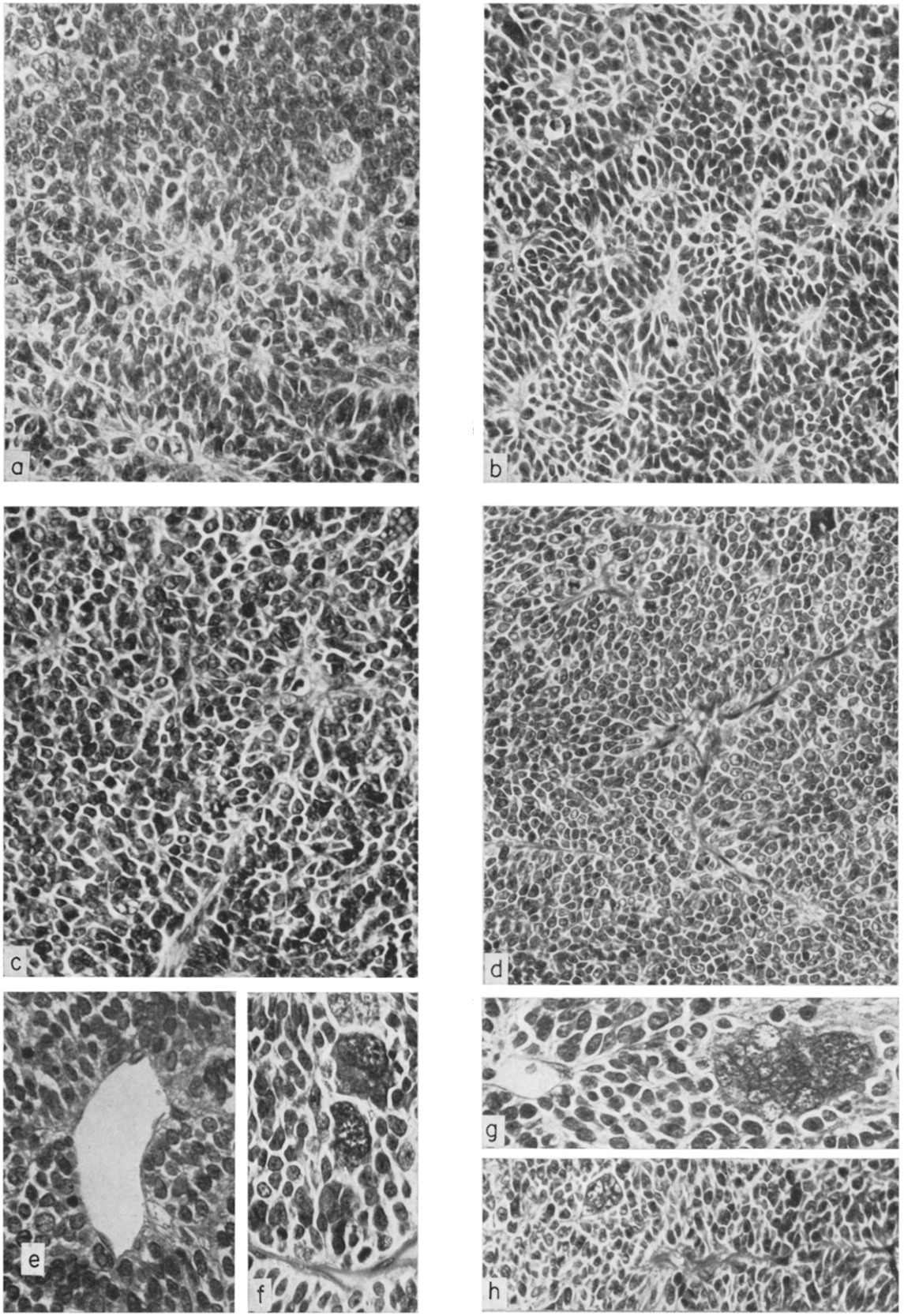


*Fig.* 1. (*a*) *Hamster adenovirus* 7 *tumor.* ×240 *e.–e. Neoplastic cells arranged in trabecular manner with pseudorosette formation. Clone of round neoplastic cells* (*upper*) *not found in PARA-adenovirus tumors. Frequent mitosis.* (*b*) *PARA-adenovirus* 7 *tumor with adenovirus T antigen.* ×240. *e.–e. Poligonal and spindle neoplastic cells arranged in trabecular manner with pseudorosette formation. Frequent mitosis.* (*c*) *PARA-adenovirus* 7 *tumor with Adenovirus T antigen.* ×240. *e.–e. More pleomorphic neoplastic cells in a less differentiated trabecular manner with rare pseudorosette formation. A giant cell in right upper corner.* (*d*) *PARA-adenovirus* 7 *tumor, without adenovirus T antigen.* ×240. *e.–e. Several layers of neoplastic cells arranged around a thin bundle of connective tissue. Well differentiated pseudorosette formations were rarely seen in this type of tumor.* (*e*) *Same section as Fig.* 2*b.* ×360. *e.–e. Ectasic capillaries with well developed wall, found in the middle of the tumor.* (*f*) *Adenovirus* 7 *tumor.* ×360. *e.–e. Typical adenovirus neoplastic giant cells.* (*g*) *PARA-adenovirus* 7 *tumor with Adenovirus T antigen.* ×360. *e.–e. A multinucleated giant cell with scanty eosinophilic cytoplasm.* (*h*) *PARA-adenovirus* 7 *tumor without adenovirus T antigen.* ×240. *e.–e. A giant cell of adenovirus type.*

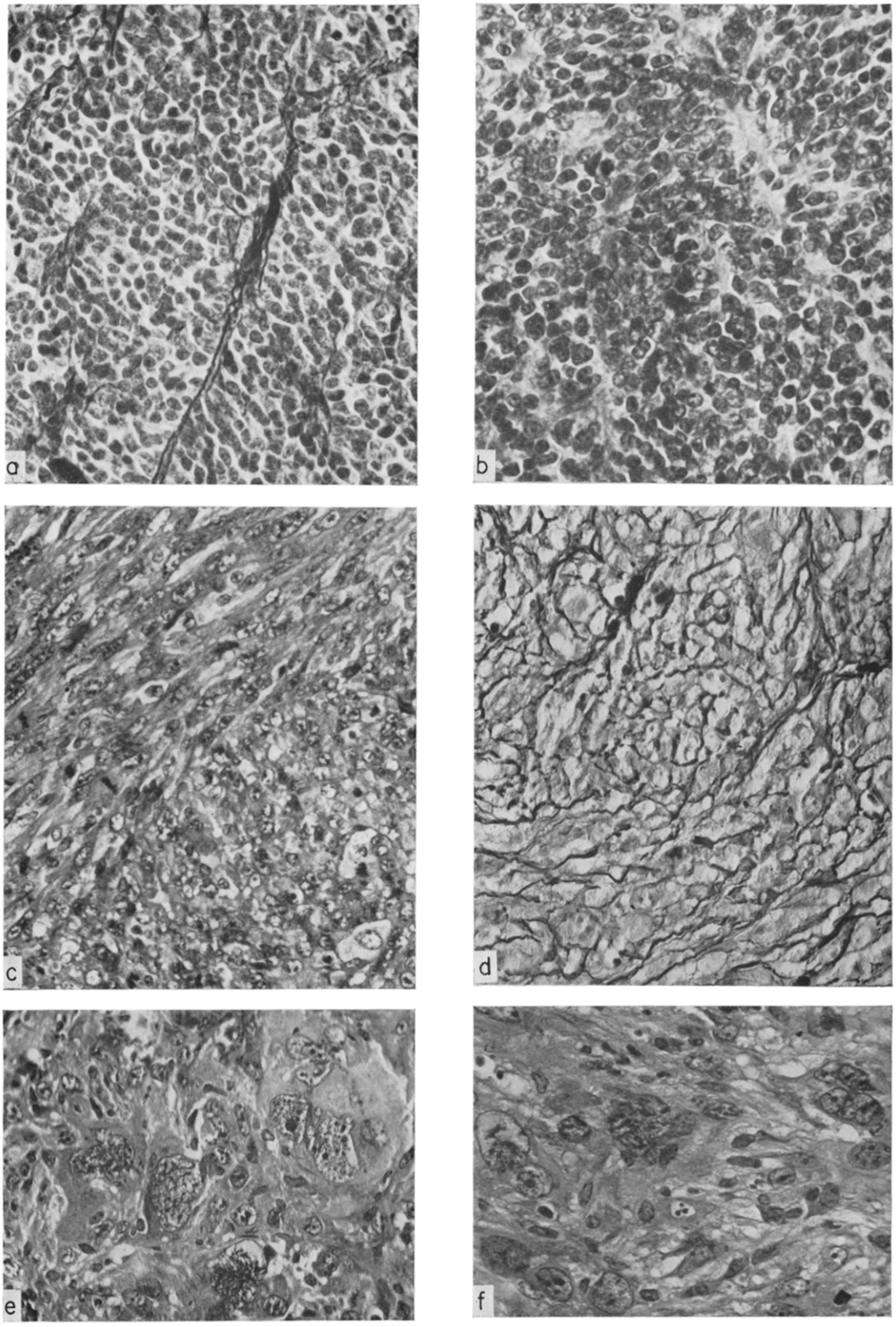


*Fig.* 2. (*a*) *PARA-adenovirus* 7 *tumor, with adenovirus T antigen.* ×240. *Silver stain (Gomori). Tiny argirophylic fibers located in a bundle of connective tissue.*

(*b*) *Simian adenovirus* 7 *tumor.* ×360. *e.–e. Less differentiated neoplastic cells with rare pseudorosette formation.*

(*c*) *SV*40 *tumor.* ×240. *e.–e. Spindle cell sarcoma. Frequent mitosis.*

(*d*) *SV*40 *tumor.* ×240. *Silver stain (Gomori). Thick net of argirophilic fibers in the intercellular neoplastic spaces.*

(*e*) *SV*40 *tumor.* ×360. *e.–e. Giant cells with large polimorphic nuclei and granular eosinophilic cytoplasm.*

(*f*) *SV*40 *tumor.* ×360. *e.–e. Cells with large nuclei and multinucleated giant cell formation.*

two or more large, round, oval or irregular shaped nuclei peripherally located and by an abundant finely granular cytoplasm (Fig. 2e and f). Although blood capillaries were numerous, foci of necrosis were very frequent. Mallory and Gomori stainings showed a thick net of thin collagen and argirophilic fibers, typical of collagen- and reticulin-producing tumors, in the intercellular spaces (Fig. 2d).

## DISCUSSION

In a previous paper [17] one of us showed that plaque-purified viral clones derived from the original PARA-adenovirus 7 induced tumors with different antigenic composition. The tumors containing both adenovirus and SV40 T antigens appeared earlier than those carrying only SV40 T antigen.

Other investigations [15] demonstrated that PARA-adenovirus 7 produces dimorphic tumors with fibrosarcoma SV40-like and undifferentiated adenovirus-like neoplastic areas in hamster. The presence of adenovirus-like tumor tissue seems to be correlated with the presence of adenovirus T antigen in the tumor.

It remained to be proved whether PARA-tumors are heterogeneous morphologically and antigenically (a) because cells transformed in a different way by the PARA-adenovirus hybrid coexist in the same tumor, or (b) because every PARA-transformed cell can differentiate to various morphologic and antigenic phenotypes.

To study this problem, two cell clones were obtained from hamster tumors induced by PARA-adenovirus 7. This virus was plaque-purified twice in order to minimize the presence of variants and mutants in its specimens. The primary tumors appeared after a latent period of less than 10 weeks; therefore they were "early" tumors which are known to contain both adenovirus and SV40 T antigens [17].

The "late" tumors, which usually contain only SV40 T antigen, were not examined. The two PARA-cell clones, 819/R/A2a and 928/R/B/Str, were isolated from the first passages *in vitro* of the tumor cells. Both clones induced transplanted tumors similar to primary or transplanted tumors induced by adenovirus 7, adenovirus 12 and SA 7 and quite different from SV40 tumors. Even after a careful examination of several specimens, no SV40-like fibrosarcoma area was observed. The histologic pattern disclosed undifferentiated small-cell tumors with abundant hemorrhagic necrosis and with some pseudorosettes, sheets of palisading tumor cells and fairly typical multinucleate giant cells. Newly formed collagen and reticulum fibers were not observed, just the opposite of H-50 cell tumors. In spite of the morphologic identity of the tumors induced by both PARA-transformed cell clones, a different antigenic pattern was shown. The cell clone 928/R/B/Str revealed both adenovirus T and SV40 T antigens at the immunofluorescent test, whilst 819/R/A2a cells showed only SV40 T antigen. When present, SV40 T and adenovirus T antigens were found in almost 100% of the cells. Even after several passages *in vitro* these antigenic characteristics of the two cell clones were unvaried (Table 2) and may therefore be regarded as relatively steady.

Rowe [24] also found that tumors induced by different strains of adenovirus type 7 differ markedly in their content of FA-stainable antigen. The Ad 7 cell strain examined by us was derived from a hamster tumor induced by adenovirus 7 and it was also devoid of FA-stainable adenovirus T antigen(s). The PARA-adenovirus 7 transformed cells may show the same inconstancy of FA-stainable adenovirus T antigen(s). The absence of such antigen(s) is however consistent with a typical adenovirus-like morphologic pattern of the transplanted PARA-tumors.

The uniform morphology of tumors induced by the two PARA-cell clones examined and the antigenic constancy of such clones do not indicate that PARA-transformed cells can differentiate into various morphologic and antigenic phenotypes easily. Therefore it is likely that cells which differ in their morphologic potency or in their antigenic composition coexist in the same PARA-tumor from the moment the oncogenic process begins.

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## SUMMARY

*Two cell clones were obtained from PARA-adenovirus 7 tumors in hamster. These cells carry SV40 T antigen, but only one carries FA-stainable adenovirus T antigen(s). These antigenic properties were found to be stable, even after several passages* in vitro.

*Both PARA-cell clones were able to induce transplantation tumors characterized by the uniform histopathologic pattern of the undifferentiated adenovirus neoplasms in hamster.*

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# The Incidence and Significance of Epithelioid and Sarcoid-like Cellular Reaction in the Stromata of Malignant Tumours. A Morphological and Experimental Study*

A. LLOMBART Jr. and J. MARTÍNEZ ESCUDERO
*Section of Experimental Cancerology, Department of Pathology, Faculty of Medicine, Valencia, Spain*

A STROMA reaction may be defined for our purposes as the sum total of morphological changes which occur in the connective tissue of an infiltrating neoplasm or in its lymph nodes.

One of the types of stroma reaction (SR) which have attracted rather special attention is the appearance of epithelioid infiltrates and also of diffuse, trabecular or nodular giant cells [1, 2] in the stratum of the tumour. This manifestation is regarded as a 'stroma reaction to epithelioid cells'. Years ago, analogous manifestations were observed by Herxheimer [3] in the lymph nodes draining the tumour and subsequently classified as 'sarcoid-like lesions' by Nickerson [4] and Symmers [5]. The incidence and significance of this reaction are still in doubt. It is generally believed that this reaction is connected with degenerative stages of the tumour—necrosis, the presence of free lipids, keratosis, irradiation etc. [2, 5, 6]—even though, in other cases [7, 8, 9] it may be an indirect morphological manifestation of a defensive immune response to the neoplasm.

This paper deals with the nature of the stroma reaction to epithelioid cells from a morphological and experimental point of view, and discusses the incidence and distribution of these structures in the neoplastic stroma of carcinomata and sarcomata in human beings (1228 cases) as well as in the lymph nodes draining carcinomatous tumours (410 cases). In addition, its possible appearance in the supporting stroma of distinct transplantable tumors in the Wistar rat and the golden hamster was investigated.

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## MATERIAL AND METHODS

*Human tumours*

One thousand two hundred and twenty-eight human malignant tumours of different types were studied histologically (Table 1). All the neoplasms reviewed were obtained by biopsy; and the number of specimens which were analysed varied, depending on the case, from four to eight. Each specimen was embedded in paraffin and then stained with hematoxylin and eosin; reticulum of Gomori and van Gieson.

The 410 lymph nodes were taken from carcinomatous, either glandular or epidermoidal, drainage areas (carcinomata of the breast, gastro-intestinal tract and of the skin and stratified mucosa). They, too, were embedded in paraffin and stained by techniques analogous to those already described.

*Experimental grafted tumors*

Six different types of transplantable tumors were grafted in groups of 20–30 animals the biological characteristics of which were known

*Table* 1. *Human material*

| Primary human tumours | Cases | Total |
|---|---|---|
| Cervix carcinoma | 180 | |
| Larynx and lung carcinoma | 185 | |
| Carcinomas of skin and pluristratified mucosa | 153 | |
| Digestive-tube adenocarcinoma | 205 | |
| Mammary carcinoma | 150 | |
| Primary and metastatic malignant liver tumours | 50 | |
| Urinary-bladder carcinoma | 20 | |
| Prostatic-gland adenocarcinoma | 15 | |
| Testicular and ovarian malignant tumours | 10 | |
| Malignant kidney tumours | 10 | |
| Sarcoma of soft tissues | 100 | |
| Malignant bone tumours | 150 | 1228 |
| Lymph nodes regional to carcinoma | 410 | 1638 |

in our laboratory. Three of these types of tumours were carcinomata — Walker 256, N-13A hepatoma and N-13R$_2$ hepatoma [10]; and the other three were sarcomata — TTS [11], ABR [10] and renal tumour induced in the golden hamster by diethylstilbestrol [12]. The implantations were carried out in the testicular passage, in keeping with the opinion of several workers [2, 13, 14] that epithelioid reaction is more pronounced in this organ.

Walker 256 carcinoma as well as N-13A and N-13R$_2$ hepatomata and ABR sarcoma were implanted in Wistar rats percutaneously by means of a trocar; and, as already mentioned, the TTS and renal sarcomata were induced in the golden hamster. Every five days after the implantation of the neoplasms, the site of the graft was subjected to direct testicular palpation as a means of determining neoplastic development. Depending on the rapidity with which the tumour grew, the animals were killed between ten and thirty days after the implantation. Both the testis and the primitive iliac lymph nodes were studied histologically, using identical techniques of embedment as for the human tumours.

## RESULTS

*Human material*

The incidence of the reaction varied and depended on the morphology of the tumour; but this finding was infrequent in general (Table 2). We have divided the reactions into the two following distinct groups: (i) relative positive and (ii) restricted positive reactions. These two terms may be defined as follows: all of the cases observed by us belong to the former group, quite independent of their morphology or connection

*Table* 2. *Related causes*

| | No. of cases | Relative positives reactions | Keratin | Necros. | Colloid | Irradiat. | Others | Restricted positives |
|---|---|---|---|---|---|---|---|---|
| Carcinomata of skin pluristratified mucosa | 153 | 20/13% | 11 | 7 | — | — | — | 2/1·3% |
| Cervical carcinoma | 180 | 14/7·7% | 5 | 4 | — | — | — | 5/2·7% |
| Larynx and lung epitheliomata | 185 | 9/4·9% | 3 | 4 | — | — | — | 2/1·0% |
| Digestive-tube neoplasms | 205 | 10/4·8% | 2 | — | 4 | — | — | 4/1·9% |
| Mammary carcinoma | 150 | 5/3·3% | — | 3 | — | 1 | — | 1/0·6% |
| Primary and metastatic hepatoma (liver neoplasm) | 50 | 1/2% | 1 | — | — | — | — | — |
| Malignant bone tumours | 150 | 1/0·7% | — | — | — | — | 1 | — |
| Sarcoma of soft tissues | 100 | 0 | — | — | — | — | — | — |
| Urinary-bladder carcinoma | 20 | 1 | 1 | — | — | — | — | — |
| Prostatic-gland carcinoma | 15 | 1 | — | — | 1 | — | — | — |
| Testicular and ovarian carcinoma | 20 | 1 | — | 1 | — | — | — | — |
| Kidney neoplasms | 10 | 0 | — | — | — | — | — | — |
| Regional lymph nodes | 410 | 10/2·4% | 2 | 3 | 2 | — | 1 | 2/0·4% |
| Total | 1638 | 73/4·4% | 25 | 21 | 7 | 1 | 2 | 16/0·9% |
| Total without lymph nodes and sarcomata | 1228 | 63/5·6% | 23 | 18 | 5 | 1 | 1 | 14/1·4% |

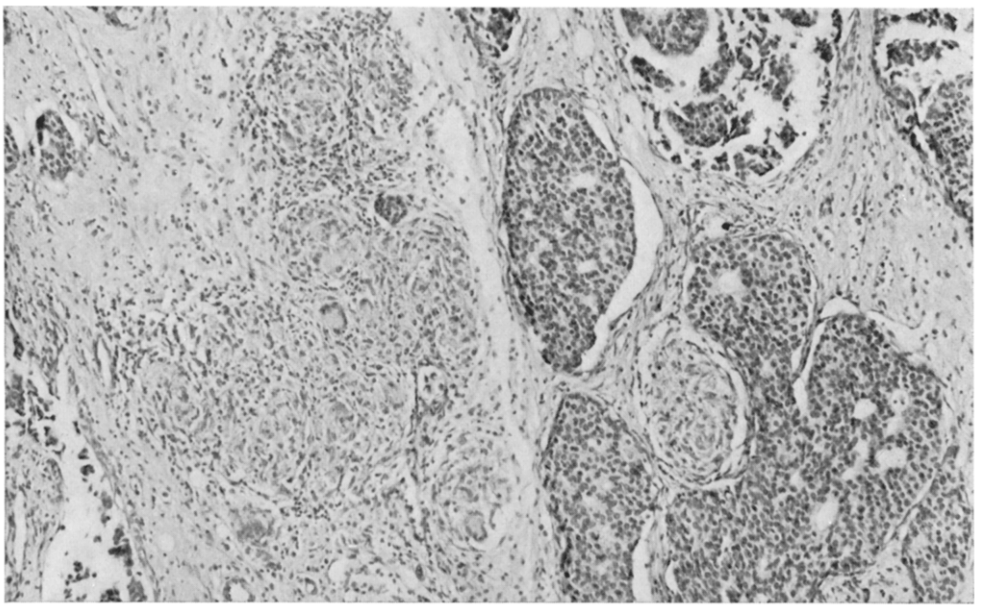

*Fig.* 1. *Mammary carcinoma* (8.10.621). *Sarcoid-like infiltrates in the stroma of the tumour. H.E.* × 20.

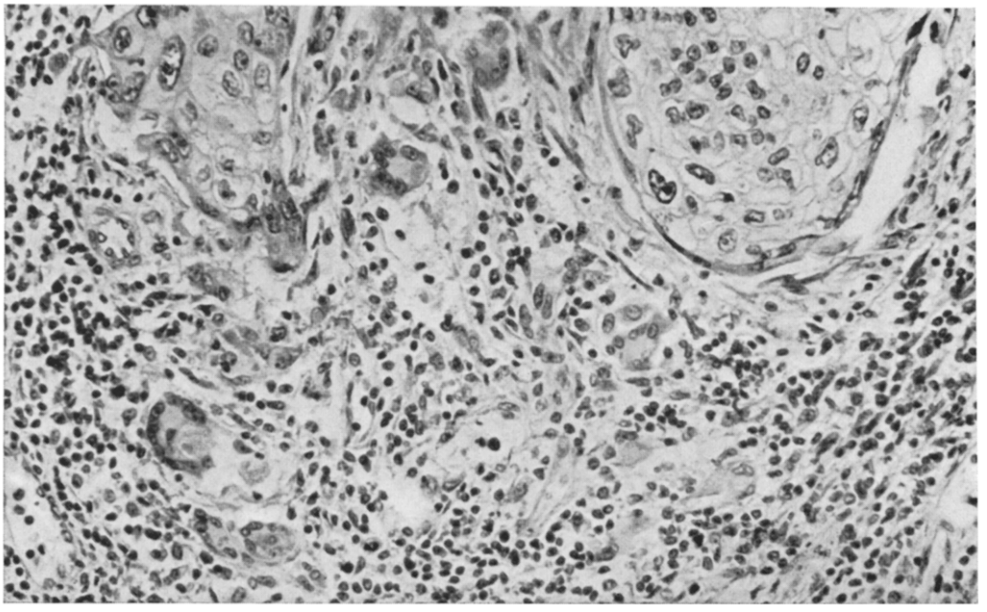

*Fig.* 2. *Positive stroma reaction of diffuse type with numerous giant cells. Mammary carcinoma.* 8.15.753). *H.E.* × 45.

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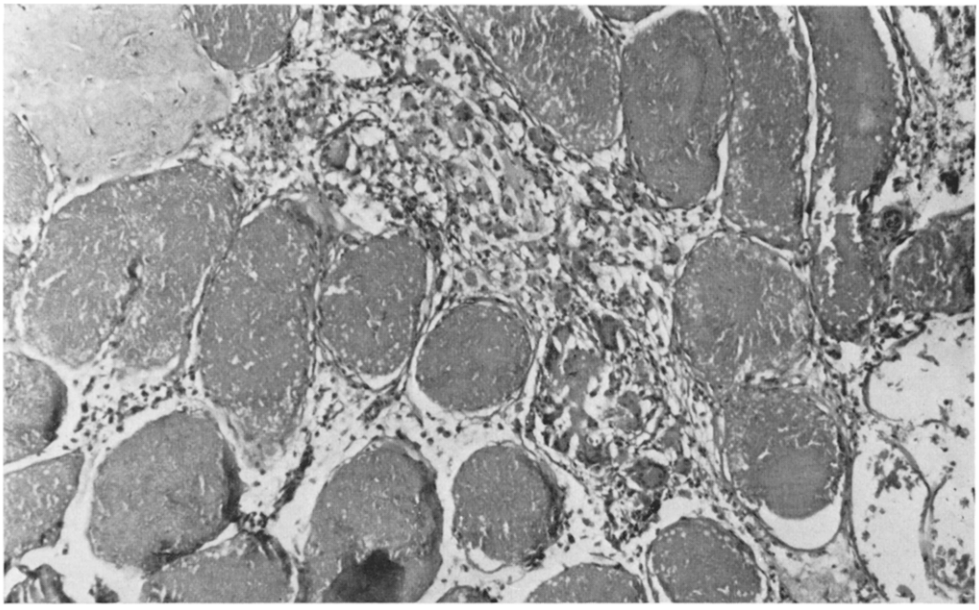

*Fig. 3. ABR fibrosarcoma grafted intratesticularly. Nodular stroma reaction with several giant cells. Tubular necrosis and interstitial oedema. H.E.* × 20.

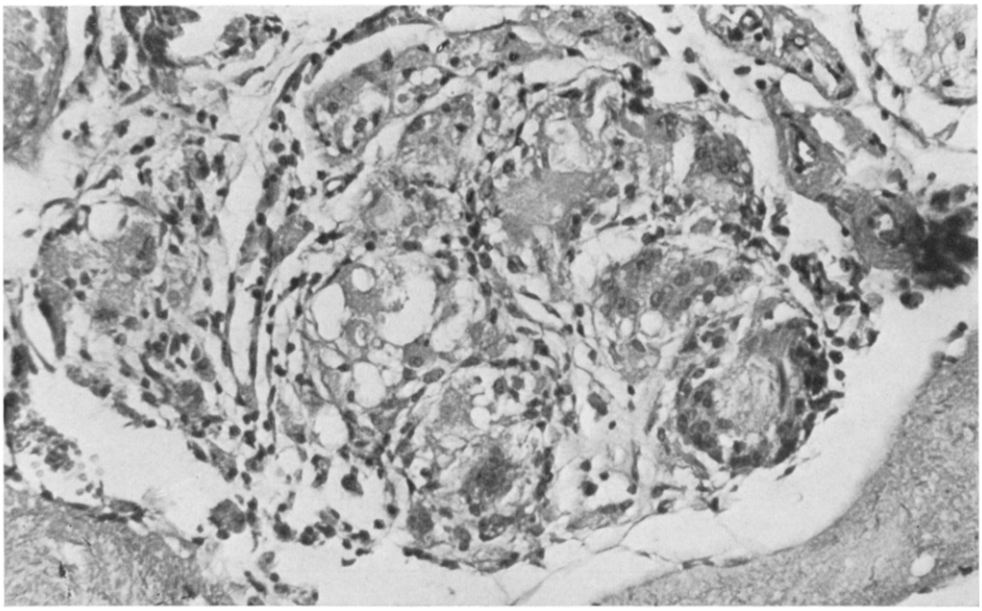

*Fig. 4. ABR fibrosarcoma grafted intratesticularly. Sarcoid-like granuloma in tubular interstitial stroma. H.E.* × 60.

with some cause that triggered off the process, whereas the restricted positives were those cases for the occurrence of which we did not find any histological justification.

*Relative positive reactions*

Carcinomata of pluri-stratified mucosa (including those of the skin) had the highest incidence (13%), followed by cervical carcinoma (7·7%), carcinoma of the lungs and larynx (4·9%), alimentary-tract adenocarcinoma (4·8%) and mammary carcinoma (3·3%). The following isolated tumours were also observed: two malignant hepatomata (metastatic), and one case each of carcinoma of the urinary bladder, prostate and testis (seminoma).

Both the soft tissue sarcomata and those which were osseous in origin were negative. We have only observed giant nodular epithelioid cells in one plasmocytoma with abundant hyaline production. The incidence in the lymph nodes of tumorous drainage was 2·4% (10 cases). In seven of these ten cases, the nodes had also been colonized by the tumour.

*Restricted positives*

The incidence of the restricted positive infiltrates was extremely low in the primary epithelial neoplasm, excluding sarcomata (1·4%), and was even lower in the lymph nodes (0·4%). The distribution based on the type of tumour was as follows: cervical carcinoma with a high level of reactivity (2·7%) followed by carcinoma of the alimentary tract (1·9%), carcinoma of pluri-stratified mucosa (1·3%), carcinoma of the lung and larynx (1%) and mammary carcinoma (0·6%).

*Histological forms and histogenesis of epithelioid cells*

We encountered most frequently the nodular type (33 cases), followed by 26 cases of the trabecular form; and typically sarcoid-like reactions were observed in six cases. An attempt has been made to reconcile these findings with those adduced by Duserre [2] and Lennert [15].

When this epithelioid reaction appeared, there were no fundamental morphological differences between the lymph nodes and the stromatic stratum. Mixed forms of sarcoid, trabecular and nodular types were observed relatively frequently in the same tumourous stratum. This fact explains why the number of types (86) exceeded the number of cases actually observed (73). We did not apply histochemical techniques which could have determined the genesis of these epithelioid cells. Some of these cells were patently macrophagic in character, whereas others, especially the multinucleate ones, seemed to originate in the vascular epithelium of granular tissue (Microphotograph No. 1). The reaction was associated with an abundant local infiltration of plasma cells, lymphocytes and, in some cases, eosinophils. They rested on a fine reticular texture but, in quite a few cases, there was diffuse fibrosis. In 46 of the 73 positive cases, there was evidence of peritumourous fibrosis.

*Experimental grafted tumours*

The results of intratesticular transplantation in experimental animals confirm the pathological findings in human beings. These results are contained in Table 3, where the presence or absence of both stromatic tumourous reaction

*Table 3. Experimental study on tumour transplants*

| Type of tumour | No. of grafted tumours | Positive implants | Negative implants | Reabsorbed tumours | Lymph nodes with metast. | Lymph nodes without metast. | Positive STR | Positive SGR |
|---|---|---|---|---|---|---|---|---|
| Walker ca. 256 (Wistar rat) | 20 | 20/0 | 0/0 | 0/0 | 17/0 | 47/0 | none | 1 case |
| N-13A hepatoma (Wistar rat) | 30 | 27/0 | 0/0 | 13/0 | 4/0 | 10/2 | none | 2 cases |
| N-13$R_e$ hepatoma (Wistar rat) | 30 | 28/0 | 2/0 | 0/0 | 0/0 | 30/0 | none | none |
| TTS sarcoma (Hamster) | 26 | 26/0 | 0/0 | 17/0 | 42/0 | 9/0 | none | none |
| ABR sarcoma (Wistar rat) | 27 | 0/0 | 27/5 | 0/0 | 0/0 | 38/0 | 5 cases | none |
| GH hormone independent tumour (Hamster) | 25 | 12/0 | 13/0 | 0/0 | 0/0 | 0/0 | none | none |

Each number of cases is followed by the number of cases of stromatic reaction.
STR—Stromatic tumoral reaction.
SGR—Stromatic ganglionary reaction.

(STR) and stromatic ganglionary reactions (SGR) is also indicated.

Walker 256 carcinoma developed in all cases (i.e. 100%), and there was a high incidence of metastasis (26·5%) in lumbo-aortic ganglia. Epithelioid cellular reactions appeared neither in the tumourous testicular stroma nor in the lymph nodes. One lymph node (of the 47 which were not colonized by the tumour) manifested epithelioid reaction with giant cells. Ascitic N-13A hepatoma inclined to reabsorption after being grafted intratesticularly. The animals which were killed ten days after implantation (14 rats) manifested the presence of testicular tumour without nodular or diffuse peripheral epithelioid reaction. The 13 rats which were killed from the 20th day after the graft onwards manifested focal necrosis without specific evidence of a stromatic reaction. Fourteen of the lymph nodes of these animals could be viewed microscopically. Metastasis occurred in four of these but without SR of epithelioid cells. There were no metastases in the remaining ten, and this latter number included two cases which were SR positive. Histologically, solid N-13$R_2$ hepatoma was colloidal in character and thus seemed suitable for triggering off this type of epithelioid reaction in the testes. Nevertheless, the actual result obtained was not affirmative. A stromatic reaction did not occur in the proximal testicular tissue of any of the 26 positive implantations. Nor did it appear in any of the 30 lumbo-aortic lymph nodes studied and which did not present metastases.

Reviewing the evidence furnished by these cases of transplanted carcinoma, we are in a position to state that, of the 75 primitive tumours which developed, none caused epithelioid reactions in the testes. Nor could the reabsorption of thirteen of them be the factor which caused this reaction. In addition, two tumours which did not 'take' were also negative. SR did not occur in the 21 metastasized lymph nodes. None the less, SR did appear in three metastasis-free nodes of the 87 which were studied (3·4% were GSR positive).

The results obtained with the group of transplanted sarcomatas — TTS, ABR and golden hamster (GH)—were as follows: *TTS sarcoma* (*Fusocellular sarcoma*). SR did not follow implantation in the 26 animals, although 20 days after the grafting, there were signs in 17 animals of tumorous reabsorption with total lysis of the graft and a focal residue of necrotic cells. Equally, SR did not appear in the 42 nodes infiltrated by sarcoma, and the same thing occurred with the nine non-metastasized nodes.

*ABR sarcoma* (*fibrosarcoma*) did not develop in any of the 30 animals in which it was implanted. Histological examination of the sites of implantation revealed, next to foci of tubular testicular necrosis, granulomatous reactive areas which were sarcoid in type and contained numerous giant cells (Microphotograph No. 2) in five cases (18·5%). On the other hand, this granulomatous reaction was not present in any of the 38 lymph nodes which were examined.

We come, last of all, to the *GH sarcoma*. Here, too, the findings were negative both with regard to the animals with the tumour (12 cases) and to those in which the graft did not 'take' (13 cases). At the same time, however, the lymph nodes of these animals were not studied.

Reviewing the evidence yielded by the implanted sarcomata, we can point to one negative SR at the level of the tumorous stratum when the graft actually 'took'. On the other hand, a nodular sarcoid-like reaction appeared in the strata of those negative implantations in five cases (that is, 11% of all sarcomata). The lymph nodes, whether or not they were colonized by these tumours, did not produce an epithelioid stromatic reaction.

## DISCUSSION

Different types of alterations at the site of the infiltration of the tumour, both in human beings and lower animals, have been regarded as stromatic reactions which have been triggered off by the malignant growth. These changes have also been regarded as a possible host reaction, both as a means of defence and in the sense of a more potent biological aggressiveness. The following have been postulated as manifestations of a local stromatic reaction: the appearance of a neobasal growth (I), the reticular isolation of the carcinomatous fibres [16–21], the presence of interstitial metachromatic oedema [19, 20, 22, 23], sclerosis of tumorous connective tissue [24, 25] and, finally, the existence of epithelioid, nodular or sarcoid infiltrates [1, 2, 13]. With regard to lymph nodes of carcinomatous drainage, the problem has been amply dealt with by numerous specialists in morphology [15, 28, 29, 30] on the level of what has been called 'sinus histiocytosis' [26]. Nevertheless, the matter remains under discussion. This is also true of sarcoid-like nodular infiltrates which are present in lymph nodes of tumorous drainage. This profile has been known of since the second decade of the present century [3]. This has recently been termed 'sarcoid-like lesions' by Symmers [5], and other workers have called

attention to the matter [6, 7, 9, 13, 28, 31]. The postulate of an active role as a defence mechanism against the tumour has been supported by Refven [7], Roulet [8], Wuketich [9], Cabanne *et al.* [13] and Heinzmann [32].

On the other hand, other workers such as Siegmund [33], Lennert [15] and Willis [30], while not expressing an explicit opinion as to its significance, regard it as being related to local degenerative processes of varying etiology as, for instance, irradiation, necrosis, parakeratosis, mucoid substances, or contamination due to mycotic or bacterial agents. Its prognostic value is therefore questionable.

The results of our experiments, which were based on a large number of primary neoplasms (1228 cases) and lymph nodes of carcinomatous drainage (410 cases), are more reconcilable with the opinion of those who attribute the reaction to causative degenerative factors than with the viewpoint which regards it as an immunologically important defensive organic reaction. The incidence observed by us (relative positive reactions) approximated to that observed by other workers who have dealt with the matter both in the strata of malignant epithelial neoplasms (5·6%) and in the regional drainage nodes (2·4%). None the less, the majority of such reactions seem to be related to kerato-necrotic areas, colloidal material or tumours which were previously irradiated in such a way, that in only 1·4% of the tumourous strata, diffuse, nodular or sarcoid-like epithelioid foci did appear, that lacked a plausible morphological explanation. The findings in the lymph nodes were analogous The relative positive reactions was 2·4%, and, if triggering-off factors (restricted positives) are excluded it was as low as 0·4%.

Having regard to the foregoing, it is clear that, while the appearance of epithelioid infiltrates of an idiosyncratic histological configuration in a certain number of cases cannot be gainsaid, the incidence of this appearance is extremely low (a maximum of 2·7% for cervical carcinoma and a minimum of 0·6% for mammary carcinoma). Its significance is therefore questionable. In the majority of cases, the epithelioid reaction was triggered off either by pathological tumourous secreton or by involutional phenomena resulting from this.

This hypothesis has been confirmed experimentally. The testicular stratum seems to offer optimum conditions for triggering off a reaction in the epithelioid cells. This response was obtained by the topical injection of certain lipidic substances [34], and lecithins [2, 35] just as in testicular tumours (seminomata and geniomata) in which, according to Masson [1], there is a higher incidence of such reactive cells. It was not possible to confirm all these hypotheses by means of locally implanting different types of experimental carcinoma (Walker 256 carcinoma, and N–13A and N-13R hepatomata). These forms of stromatic reaction appeared neither in the tumours which 'took' nor in those which did not. The incidence of stromatic reaction with giant cells was 3·4% for the 87 metastatic and non-metastatic nodes which were examined and which came from lumbo-aortic drainage areas. None of these nodes was colonized by the tumour, and the values approximated to those observed in tumours in human subjects.

When they developed, the sarcomata injected intratesticularly (ABR, TTS and GH) did not give rise to an epithelioid reaction in the local stromata or in the lymph nodes. Here, our findings are in agreement with those in human subjects, since, of 250 soft tissue and skeletogenous sarcomata, sarcoid-like nodules were produced in only one plasmocytoma associated with severe paraproteinosis.

In the ABR sarcoma, which was systematically rejected at testicular level, nodules appeared in five cases; and these nodules were associated with peripheral tubular necrosis. This appearance is probably due to the exudation in the interstices of spermatic matter such as the spermatic granuloma which Mullaney has produced experimentally in rats [14]. We do not believe that this is a defence reaction of the organism of the rat against a graft which did not 'take'.

## SUMMARY

*The authors have studied, both morphologically and experimentally, the incidence and significance of epithelioid and sarcoid-like cellular reaction in* 1,228 *human tumours and* 410 *lymph nodes of carcinomatous drainage. Three types of carcinomata (Walker–*256, *N–*13*A hepatoma and N–*13$R_2$ *hepatoma), and three sarcomata (TTS, ABR, and hormone-independent GH sarcoma) were implanted in the testicles of the Wistar rat and golden hamster.*

*The incidence of this stroma reaction, free of histologically verifiable causative factors, in the tumorous area was very low* (1·4%). *The incidence of stroma reaction in the*

*sarcomata was* 0, *and in lymph nodes of carcinomatous drainage it was only* 0·4%; *there was no reasonable morphological explanation for this. These findings in human subjects were confirmed by the results of experimentally transplanting tumors intrasteticularly.*

*The epithelioid and sarcoid-like reaction of tumorous stroma seems to be mostly due to neoplastic desintegrative phenomena.*

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# Letter to the Editor

## Development of Newborn Mice During Prolonged Treatment with Interferon

I. GRESSER and C. BOURALI

*Laboratory of Viral Oncology,*
*Institut de Recherches Scientifiques sur le Cancer, Villejuif, France*

WE HAVE previously stressed the efficacy of exogenous interferon in the treatment of virus infected [1, 2] and tumour inoculated mice [3–5]. Therapeutic results were not accompanied by any significant toxicity [6, 7]. In contrast, various toxic effects have been observed in laboratory animals inoculated with non-virus inducers of endogenous interferon, such as polyinosinic-polycytidylic acid (poly I· poly C) [8–11], pyran copolymers [12] and endotoxin. The question of the possible toxicity of exogenous interferon and of interferon inducers is relevant to the interpretation of the results of animal experimentation, as well as to considerations of the eventual therapeutic usefulness of interferon in clinical and veterinary medicine. In view of the lack of published experimental data on the toxicity of exogenous interferon, it was considered of interest to determine the effect of daily inoculation of potent interferon preparations on the growth of newborn AkR and C3H mice.

The preparations of mouse interferon utilized in these experiments were extracted from the brains of Swiss and IC mice inoculated with West Nile Virus and concentrated 10-fold as previously described [1, 2]. In the experiments reported herein, newborn AkR and C3H mice were inoculated with 32,000–64,000 interferon units/day (in 0·2 ml) (expressed in International mouse interferon reference units N.I.H.). The concentration of protein was 4·3 mg/ml for the brain interferon and 3·7 mg/ml for the normal brain extract.

Litters of newborn AkR and C3H mice were distributed at random into 4 groups: (a) untreated; (b) treated with P.B.S.; (c) treated with normal brain extract, and (d) treated with brain interferon. Three experiments were undertaken, and the volume inoculated/day in groups b, c and d was as follows:

| | Day 0–day 7 | Day 7–day 28 |
|---|---|---|
| Exp. 1 | 0·05 ml | 0·10 ml |
| Exp. 2 | 0·10 ml | 0·20 ml |
| Exp. 3 | 0·15 ml | 0·20 ml |

All mice were inoculated subcutaneously in the interscapular region. Mice were weighed individually daily on a Mettler balance for the first 7 days and every other day thereafter. On the 29th day, all mice were sacrificed and autopsied. The thymus, liver, spleen and left kidney were weighed. Histologic sections of the thymus gland of all mice were examined.

No consistent difference was observed in the growth of newborn AkR or C3H mice inoculated daily with concentrated brain interferon, concentrated normal brain or P.B.S. or left untreated (Fig. 1). Although 37 litters of AkR and C3H mice were distributed into the 4 groups in the 3 experiments only 27 litters remained more or less intact by the 29th day permitting their inclusion in the experiment. The loss of one or several litters of AkR and

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C3H mice was observed in each of different groups as follows:

| | Treatment | | | |
|---|---|---|---|---|
| | None | P.B.S. | Normal brain | Interferon |
| No. of litters initially | 9 | 11 | 8 | 9 |
| No. of litters lost | 2 | 5 | 1 | 2 |

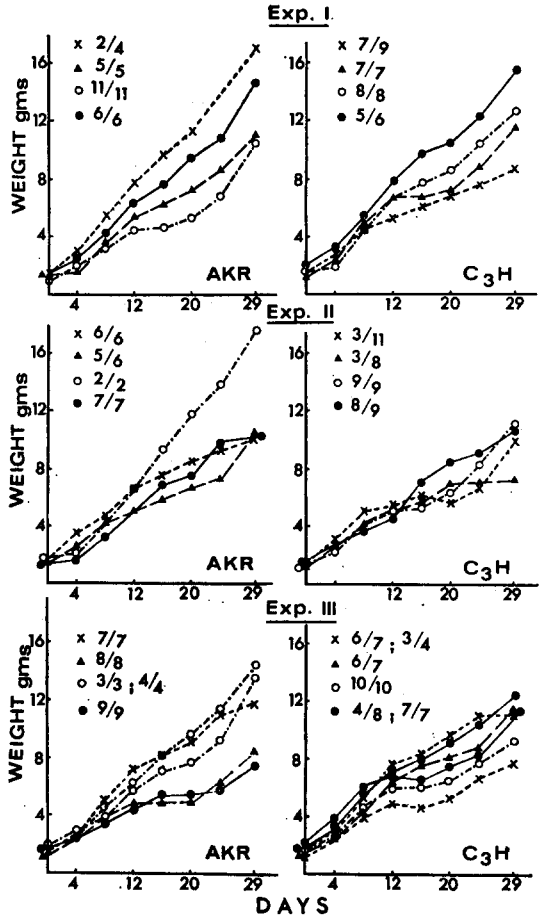


*Fig.* 1. x = *untreated mice;* ▲ = *mice treated with P.B.S.;* o = *mice treated with control brain preparations;* ● = *mice treated with brain interferon preparations.*

*There is at least one litter/treatment group in each experiment.*

*In Experiment* 3 *there were* 2 *litters of AkR mice treated with normal brain extract,* 2 *litters of C3H mice left untreated and* 2 *litters of C3H mice treated with interferon preparations.*

*The number of mice in each litter surviving to the* 29*th day/the total number of mice in the litter at birth is presented in the upper left hand corner of each graph.*

Likewise, there was no significant difference in the mortality encountered in the 27 litters, representing the different treatment groups in the 3 experiments (Fig. 1).

The mean total body weight at 29 days and the mean weight of the thymus, spleen, liver and left kidney of mice in the different treatment groups was compared and is presented in Table 1. The analysis of variance showed no significant difference for the 2 strains of mice, (a) between the 3 control groups and (b) between the control groups together compared to the interferon treated group with one exception: the mean weight of the spleens of interferon treated C3H mice was greater than the mean weight of the spleens of control mice. However, when the ratio of the weight of each organ to total body weight of the mouse was determined and compared, no significant differences were observed (and the difference previously noted for the spleen weight of interferon treated C3H mice was no longer present).

Histologic sections of the thymus of all mice were examined and no significant abnormalities attributable to treatment were observed.

In summary, the daily subcutaneous administration of concentrated brain interferon preparations was well tolerated and no evidence of toxicity was observed as determined by: (1) overall mortality; (2) the growth curve of treated mice; (3) the weight of various organs at autopsy, and (4) histologic examination of the thymus glands. It is possible that under different experimental conditions and employing different criteria, interferon toxicity might have been detected. We should emphasize however that the interferon preparations employed in these and previous investigations are to our knowledge the most potent that have been utilized in any *in vivo* experimentation. This apparent lack of toxicity of exogenous mouse brain interferon is in contrast to the reported toxicity for laboratory animals of various non-viral inducers of endogenous interferon.

It has previously been shown that interferon preparations, comparable to those utilized in these experiments, inhibited the multiplication of murine leukaemic cells *in vitro* [13] and the growth of several transplantable tumors in mice [3, 4]. Since virtually all anti-tumor drugs to date have exhibited significant toxicity in test animals, the apparent lack of interferon toxicity seems of some interest.

*Table 1. Mean total body weight and weight of various organs of mice in the 3 experiments at day 29*

| Treatment | AkR | | | | $C_3H$ | | | |
|---|---|---|---|---|---|---|---|---|
| | None | P.B.S. | Normal brain | Interferon | None | P.B.S. | Normal brain | Interferon |
| Total body weight (g) | 13± 3* | 10± 3 | 14± 3 | 11± 3 | 10± 2 | 10± 2 | 11± 2 | 12± 2 |
| Thymus (mg) | 72± 26 | 82± 26 | 113± 22 | 80± 26 | 42± 22 | 53± 26 | 59± 26 | 69± 22 |
| Spleen (mg) | 43± 24 | 52± 24 | 68± 21 | 57± 24 | 95± 26 | 109± 30 | 102± 30 | 149± 26 |
| | | | | | | NS | $p = 0{\cdot}02$ | |
| Liver (mg) | 580±209 | 517±209 | 742±178 | 640±209 | 615±139 | 595±164 | 674± 164 | 799±139 |
| Left kidney (mg) | 79± 25 | 77± 25 | 102± 21 | 80± 25 | 90± 16 | 98± 19 | 101± 19 | 114± 16 |

*0·95 confidence interval.

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# Letter to the Editor

## An Experimental Model of Active Immunotherapy Preceded by Cytoreductive Chemotherapy*

J. L. AMIEL and M. BERARDET
*Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse, 94-Villejuif, France*

NON-SPECIFIC active immunotherapy has been shown to be effective for the control of antigenic syngeneic murine leukaemias; first demonstrated for a virus-induced syngeneic leukaemia [1], then for a syngeneic leukaemia induced by a chemical carcinogen [2]. The results of these experimental studies have already been applied to devise new ways to treat acute lymphoblastic leukaemia in man [3]. Immunotherapy has only been found to be effective if the number of malignant cells in less than a critical maximum ($10^4$ cells for the murine E ♂ G2 leukaemia) [1] and is only effective when it is not followed by cytostatic chemotherapy [4]. The effects of a specific and a non-specific immunotherapy, though they are not always additive, do not oppose one another [4] and can sometimes have a mutual potentiality [5].

The experimental models studied so far differ from the clinical situation encountered in man. Active immunotherapy, whether it is given before [1] or after [2] an injection of leukaemic cells, acts on population of malignant cells which have not had time to implant themselves in privileged sites, such as the central nervous system; the immunotherapy is tested alone in normal animals given a graft of leukaemic cells, but whose immune defences have not been altered by immunosuppressive cytostatic agents [6], nor by the late effects of the leukaemic population *per se* [7].

We considered it of interest to study a model system with closer resemblance to the conditions under which active immunotherapy is used for the treatment of human leukaemia. In this model, active immunotherapy is not applied until 10 days after grafting a population of syngeneic leukaemic cells, given in a sufficiently large number not to be destroyed by the animals' immune reactions alone, even if they were stimulated [1, 8]. The immunotherapy is given after a course of chemotherapy required to reduce the number of leukaemic cells, but this also has an immunosuppressive action [9]. This model permits the action of various forms of immunotherapy to be tested on the residual population—that is, cells lodging in sites least accessible to the chemotherapy; indeed it is hoped to kill cells remaining in these sites is the function of immunotherapy in the treatment of human leukaemia.

### MATERIALS AND METHODS

Seventy F1 (DBA/2 × C57B1/6) male mice, aged 3 months, were given $10^5$ syngeneic E ♂ G2 leukaemic cells intraperitoneally on day 0; they were then divided into 5 groups of 14 mice. The mice in Group I received no treatment; the mice of the 4 other groups received, from day 4 to day 9 inclusive, 246 mg/kg procarbazine per day intraperitoneally—that is, one-

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*Table 1. Effects of an active immunotherapy preceded by a cytoreductive chemotherapy in F1 (DBA/2 × C57B1/6) mice given $10^5$ compatible E ♂ G2 leukaemic cells intraperitoneally*

| Group | No. of mice | Mice died of leukaemia | Median and limits of survival time in days |
|---|---|---|---|
| I | 14 | 14 | 16·5 (14–19) |
| II | 14 | 14 | 38·4 (34–48) |
| III | 14 | 1 | — |
| IV | 14 | 0 | — |
| V | 14 | 0 | — |

Group I: Not treated.
Group II: procarbazine, 246 mg/kg/day (day 4–9).
Group III: procarbazine + $10^7$ AkR leukaemic cells on day 10.
Group IV: procarbazine + 1 mg living BCG on day 10.
Group V: procarbazine + 0·5 mg formolized corynebacterium parvum on day 10.

third of the LD 50 at 6 days (Garattini, personal communication). On the 10th day, the mice in Group III received $10^7$ living AkR leukaemic cells subcutaneously in 0·2 ml Hank's medium; the mice in Group IV were injected intravenously with living BCG (1 mg dry wt/mouse, obtained from the Institut Pasteur); the mice in Group V were each injected intravenously with 0·5 mg formolized corynebacterium parvum (the suspension contained 24 mg of formolized corynebacterium parvum per ml).

The animals' survival was studied up to 100 days; the presence of leukaemia was checked macroscopically in every dead mouse.

## RESULTS

The results are summarized in Table 1. Procarbazine alone gave a considerable increase in the animals' survival time, but did not produce any cure. All the mice in Group II died of leukaemia; on the other hand, specific active immunotherapy (Group III), non-specific from BCG (Group IV) of formolized corynebacterium parvum (Group V) enabled all the animals, except one in Group III, to survive for 100 days without signs of leukaemia.

## DISCUSSION

The results obtained have shown that this experimental system was found to be well suited for its aim: to produce evidence of an effect of active immunotherapy under conditions which approach those encountered in the treatment of human acute leukaemia. The final outcome, death from leukaemia or cure, is affected by several factors: (1) number of leukaemic cells at the beginning; (2) duration of the growth of the leukaemia before commencing chemotherapy; (3) the effectiveness of cytoreductive chemotherapy; (4) immunosuppression during the chemotherapy and, especially, the persistence of immunosuppression after stopping chemotherapy; (5) the time interval between the end of cytoreductive chemotherapy and starting active immunotherapy; (6) the type of active immunotherapy, specific or non-specific; (7) the influence of various combinations of types of active immunotherapy.

It seems that the experimental model that we have studied enables each one of these parameters to be varied independently. However, one very important factor in the prognosis of human acute leukaemia cannot be reproduced by this experimental model, that is the localization of leukaemic cells in the central nervous system. It is known, when L 1210 leukaemic cells become localized in the central nervous system the disease can no longer be cured by usual chemotherapy or immunotherapy. It is clearly evident that prophylactic intrathecal chemotherapy plays a major role in the eventual prognosis of acute lymphoblastic leukaemias. It will be very interesting to know if the E ♂ G2 leukaemia, which is an excellent material for testing active immunotherapy, can localize in the same way as the L 1210 leukaemia and, if this is so, under what conditions it will occur and how it could be prevented.

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# Editorial Announcement

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WITH IMMEDIATE EFFECT, the Editor will accept for publication in *The European Journal of Cancer* only papers which are written in English. Will prospective authors please note that in future they should not submit manuscripts in French or German.